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RNA Editing of the Human Serotonin 5-Hydroxytryptamine 2C Receptor Silences Constitutive Activity*

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RNA transcripts encoding the serotonin 5-hydroxytryptamine 2C (5-HT_{2C}) receptor (5-HT_{2C}R) undergo adenosine-to-inosine RNA editing events at up to five specific sites. Compared with rat brain, human brain samples expressed higher levels of RNA transcripts encoding the amino acids valine-serine-valine (5-HT_{2C_VSV}) and valine-glycine-valine (5-HT_{2C_VGV}) at positions 156, 158, and 160, respectively. Agonist stimulation of the nonedited human receptor (5-HT2C_INI) and the edited 5-HT2C_VSV and 5-HT_{2C_VGV} receptor variants stably expressed in NJH-3T3 fibroblasts demonstrated that serotonergic agonists were less potent at the edited receptors. Competition binding experiments revealed a guanine nucleotide-sensitive serotonin high affinity state only for the 5-HT_{2C_INI} receptor; the loss of high affinity agonist binding to the edited receptor demonstrates that RNA editing generates unique 5-HT2cRs that couple less efficiently to G proteins. This reduced G protein coupling for the edited isoforms is primarily due to silencing of the constitutive activity of the nonedited 5-HT2CR. The distinctions in agonist potency and constitutive activity suggest that different edited 5-HT_{2C}Rs exhibit distinct responses to serotonergic ligands and further imply that RNA editing represents a novel mechanism for controlling physiological signaling at serotonergic synapses.

RNA editing is a post-transcriptional modification resulting in an alteration of the primary nucleotide sequence of RNA transcripts by mechanisms other than splicing. The enzymatic conversion of adenosine to inosine by RNA editing has been identified within an increasing number of RNA transcripts, indicating that this modification represents an important mechanism for the generation of molecular diversity. Several of these editing events have been shown to have significant consequences for cellular function. Transcripts encoding the B-subunit (GluR-B) of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid subtype of glutamate receptor undergo RNA editing events that modulate both the ion permeation and electrophysiological characteristics of this glutamate-gated ion channel (1–3). Mice that are deficient in their ability to edit GluR-B transcripts die at 3 weeks of age due to epileptic seizures, suggesting that editing of GluR-B RNA is important in the modulation of normal glutamatergic neurotransmission (4). These results suggest that the consequences of editing events within other, diverse RNA molecules might also have important ramifications for cellular function.

The monoamine 5-hydroxytryptamine (serotonin; 5-HT)[1] interacts with a large family of receptors to induce signal transduction events important in the modulation of neurotransmission (5). The 2C subtype of serotonin receptor (5-HT_{2C}R) is a member of the G protein-coupled receptor superfamily and stimulates phospholipase C, resulting in the production of inositol phosphates and diacylglycerol (6). We have recently shown that RNA transcripts encoding the rat, mouse, and human 5-HT_{2C}R undergo adenosine-to-inosine RNA editing events at five positions, termed A, B, C, D, and E (previously termed C') (7,8), resulting in an alteration of amino acid coding potential within the putative second intracellular loop of the encoded protein. Editing at the A site, or at the A and B sites concurrently, converts an isoleucine to a valine at amino acid 156 of the human receptor, while editing at the B position alone generates a methionine codon at this site (Fig. *1A*). Editing at C converts asparagine 158 to a serine; editing at E generates an aspartate at this site, and conversion at both C and E generates a glycine triplet. Editing at D results in the substitution of a valine for an isoleucine at position 160.

We have previously demonstrated a decrease in 5-HT potency when interacting with the rat 5-HT_{2C}R isoform 5-HT_{2C_VSV}, which is simultaneously edited at the A, B, C, and D positions encoding valine, serine, and valine at positions 157, 159, 161, respectively. This decrease in potency was reflected as a rightward shift in the dose-response curve for [^{3}H]inositol monophosphate accumulation (7). We proposed that the decreased potency resulted from a reduced G protein coupling efficiency induced by the introduction of novel amino acids into the second intracellular loop, a region known to be important for G protein coupling (9–16). In the present study, we show that the patterns of 5-HT_{2C}R editing differ in human *versus* rat brain, resulting in the generation of novel receptor isoforms in human tissue. Radioligand binding and functional studies were designed to more fully test the hypothesis that the decreased agonist potency of edited isoforms reflects alterations in G protein coupling. The results show that differences in the ability to spontaneously isomerize to the active R* conformation

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[1] The abbreviations used are: 5-HT, 5-hydroxytryptamine; 5-HT_{2C}R, 5-HT_{2C} receptor; peR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; DOI, (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane; DMT, *N,N*-dimethyltryptamine; LSD, lysergic acid diethylamide.

playa role in the altered coupling properties of the fully edited receptor, suggesting that RNA editing of the 5-HT$_{2C}$R represents a novel mechanism for regulating neuronal excitability by stabilizing receptor signaling and enhancing the signal: noise ratio at serotonergic synapses.

EXPERIMENTAL PROCEDURES

Preparation of RNA, Reverse Transcription-peR, and Editing Efficiency Analyses—Total RNA isolated from whole human brain was obtained from CLONTECH (Palo Alto, CA). Reverse transcription-peR amplification of 5-HT$_{2C}$R messenger mRNA was performed as described previously (7) with the antisense oligonucleotide 5'-GCAGTAACAT-CAAAGCTTGTCGGCG-3' (coordinates 723-747 relative to the translation start site of the human 5-HT$_{2C}$R eDNA; Ref. 17) employed for eDNA synthesis. The sense oligonucleotide primer 5'-CCAGGG-AAT-TCAAACTTTGGTTGCTTAAGACTGAAGC-3' (coordinates −80 to -5; Ref. 18) and the original eDNA synthesis primer or, in some cases, 5'-ATTAGAATTCTATITGTGCCCCGTCTGG-3' (coordinates 372-389; Ref. 17) and the cDNA priming oligonucleotide (boldface sequence indicates introduced *EcoRI* restriction sites) were used for peR amplification of human 5-HT$_{2C}$R sequences. Amplification proceeded for 75 s at 94 °C, for 75 s at 50 °C, and for 150 s at 72 °C for 35 cycles. Products were purified on a 2% agarose gel, and primer extension analyses at the A and C/D sites were performed as described (7). For sequencing analysis, PCR fragments were digested with *EcoRI* and *HindIII* and lmidireetionally subcloned into pBKSU- {Stratagene). Individual eDNA isolates were sequenced using the fmole sequencing system (Promega) as described previously (7).

Preparation of Human 5-HT$_{2C}$R Isoform DNA and Expression in Cell Culture—Human 5-HT$_{2C}$R variants were prepared by oligonucleotide-directed mutagenesis of human INI receptor DNA (a gift of Dr. Alan Saltzman) as described previously (10, 19). These constructs were unidirectionally cloned into the eukaryotic expression vector pCMV2 (a gift of Dr. David Russell) at the *EcoRI* and *XbaI* sites. Transient experiments in NIH-3T3 cells were performed as described previously (7). For transient expression into the COS-7 edt line, cells were plated in 24-well plates at a density of 1 x 10^5 cells/well 24 h prior to transfeetion. Two μl of Lipofectamine reagent® (Life Technologies, Inc.) and 0.5 μg of plasmid were combined in 400 μl of serum-free DMEM and added to each well for 5 h. Cells were washed with phosphate-buffered saline, and complete medium (DMEM, 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin) was added for 24 h. Stable cell lines were generated by co-electroporation of human isoforms in pCMV2 (60 μg) and pRC/CMV (Invitrogen, Carlsbad, CA) (6 μg) using electroporation conditions described previously (7). Single clones were selected using 1 mg/ml G418 <Geneticin®) in DMEM containing 10% dialyzed calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 1% calf serum (Hydone®; Hyclone Laboratories, Logan UT). Cells were maintained in DMEM with 10% calf serum (Hyclone®), 100 units/ml penicillin, 100 μg/ml streptomycin, 500 μg/ml G418, and 1 μM 2-bromolysergic acid diethylamide.

Phosphoinositide Hydrolysis Assay-Transiently transfected cells were prepared for inositol monophosphate analysis as described previously (NIH-3T3 cells, 7; COS-7 celis, 21). For stable ceHlines, cells were washed three or four times with Hanks' buffer prior to plating in a 24-well plate (Falcon 3047, Becton-Dickinson Laboratories, Lincoln Park, NJ) in DMEM containing 10% dialyzed calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. 24 h later, cells were labeled for 16-20 h with 1 μCi of *myo*-[^{3}H]inositol/ml (20-25 Cilmmol, NEN Life Science Products) in serum-free, inositol-free DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin. For isolation of inositol monophosphates from both transient and stable iransfectants, agonists were added in the presence of 10 mM lithium chloride and 10 μM pargyline. Incubations continued for 30 min unless otherwise noted. [3HJInositol monophosphates were isolated as described previously (22). Concentration response curves were analyzed using GraphPad Prism® software (GraphPad Software Inc., San Diego, CAI.

Radioligand Binding-Radioligand binding analyses were performed in transiently transfected cells as described previously (7,21). Stable cell lines were prepared by washing thl'ee or four times in Hanks' buffer and replacing the medium with D:MEM containing 10% dialyzed calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin for 24 h. Subsequently, cells were placed into serum-free DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin for 16-18 h prior to analyses. PHlMesulergine binding was assayed in crude membrane preparations in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5, as described previously (23). Competition binding was performed with 0.5 nM [^{3}H]mesulergine in the presence of increasing concentrations of nonlabeled competitor at 37 °C fol' 30 min. In some experiments, 100 μM guanosine 5'-({3.-y-imido)triphosphate (Gpp(NH)p) was added during the incubation. IC_{50} values were determined by fitting data to a sigmoidal curve with variable slope using GraphPad Prism® (GraphPad Software, Inc., San Diego, CAl; one-site and two-site binding curves were compared using the *F* ratio. IC_{50} values were converted to K_i using the transformation of Cheng-Prusoff (24).

RESULTS

The 5-HT$_{2C}$R Editing Pattern in Human Brain Is Distinct from Rat Brain—To determine the editing pattern for 5-HT$_{2C}$R transcripts in human brain, whole brain total RNA was amplified by reverse transcription-PCR. The extent of editing at specific sites and the editing patterns within individual RNA species were determined by primer extension analysis and by subcloning and sequencing individual cDNA isolates, respectively. These studies revealed that the extent of editing in human brain at the A and D positions was similar to the levels previously identified within rat brain RNA, while editing at position B was lower in human brain (Ref. 7; Fig. IE). By contrast, the editing efficiency at the C position was much higher in 5-HT$_{2C}$R transcripts isolated from human compared with rat brain (60 *versus* 35%). Editing at the E position (previously referred to as C') was barely detectable in rat brain mRNAs from a variety of brain regions «5%; Ref. 7), but this position was edited with much higher efficiency in human brain transcripts (Fig. IE).

Due to increased levels of editing at the C and E positions, the pattern of editing observed within individual human brain 5-HT$_{2C}$R transcripts differed from that found in the rat (Fig. IC). For example, the 5-HT2C.VSV isoform, which comprised approximately 10% of the 5-HT$_{2C}$R transcripts isolated from whole· rat brain, represented almost 40% of the messages derived from whole human brain (Fig. IC). The enhanced editing at the E position in humans was also reflected by the appearance of variant RNAs encoding the novcl5-H'f2C_VGV' 5-HT2C_vGJ' and 5-HT$_{2C\cdot IGI}$ protein isofonns (Fig. IC). RNA encoding the 5-HT$_{2C\text{-}VNV}$ variant, which represented approximately 50% of the transcripts isolated from rat brain (7), only comprised 8% of the total5"HT$_{2C}$R RNAs found in human brain (Fig. ICl. Together, these results indicated that editing of the 5-HT$_{2C}$R RNA differs considerably between rodent and human species and further suggested that the isoforms specific to human brain may have distinct functional roles_

Differential Signaling Profiles of the Human Edited Receptor Isoforms-To assess the functional consequences of editing within human 5-HT$_{2C}$R RNAs, we transiently expressed six of the major human isoforms in NIH-3T3 fibroblasts and examined the ability of these receptors to stimulate the phospholipase C signal transduction cascade by measuring the accumulation of [^{3}H]inositol monophosphates. Results from these studies revealed that the human 5-HT2C_VSV receptor exhibited a 5-fold shift in potency for 5-HT compared with the5-HT$_{2C\cdot INI}$ receptor (Fig. 2). The 5-HT$_{2C\text{-}VGV}$ receptor exhibited a more substantial rightward shift in the dose-response curve for 5-HT, with an EC_{50} value of 59 *versus* 2.3 nM for the 5-HT2C_INI receptor variant. The EC_{50} values for all other isoforms were not significantly different from the EC_{50} observed for the 5-HT$_{2C\text{-}INI}$ receptor.

To more carefully examine the properties of the human edited receptors, stable cell lines of the human 5-HT$_{2C\text{-}INI}$ 5-HTzc.vsv, and 5-HT$_{2C\text{-}VGV}$ receptor isoforms weregenel'ated in NIH-3T3 cells. The density of receptors in these cell lines was 258 ± 87 for 5-HT$_{2C\text{-}INI}$, 173 ± 28 for 5-HT2C_VSV' and 1054 ± 260 fmollmg protein for the5-HTZC_VGV Lsoform. Similar to the phenotype observed in the transient transfections, 5-HT exhibited a lower potency when interacting with either

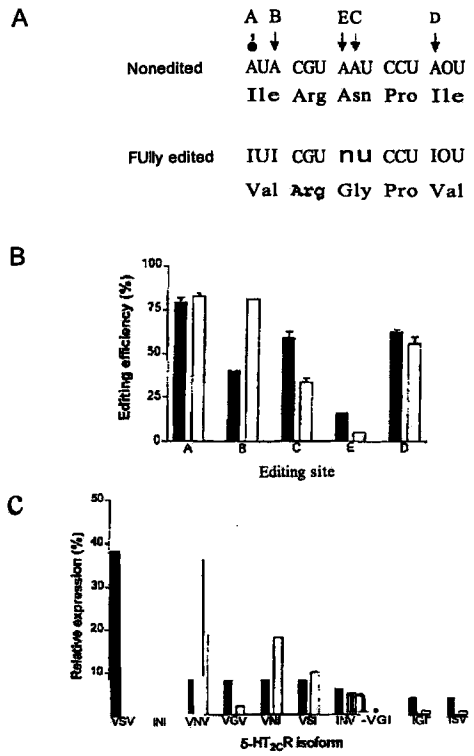

FIG. 1. 5.HT$_{2C}$R editing efficiencies in human *versus* rat brain. *A*, the positions of the editing sites within human 5-HT$_{2C}$R RNA are shown with the nonedited RNA sequence at the *top* and the fully edited sequence indicated at the *bottom*. The positions afthe five editing sites, encompassing amino adds 156-160 of the human 5-HT$_{2C}$R sequence are shown. *B*, the editing efficiency at each editing site for whole human <*black bars)* and whole rat *(white bars)* brain is shown. Editing efficiendes at the A, C, and D sites were determined by a combination of primer extension (n = ≥2 independent analyses) and sequencing analyses (n = 50 independent cDNA dones for human samples); values represent the mean ± S.E. Editing efficiencies at the Band E sites were determined by sequencing of 50 independent cDNA clones from human brain. C, the relative expression level, presented as a percentage of total isoform expression derived from a sequencing analysis of 50 (human) or 100 (rat) brain cDNA dones, is compared for 5-HT$_{2C}$R RNA derived from human *(black bars) versus* rat *(white bars)* brain. Rat data are from Burns *et al. (7)*.

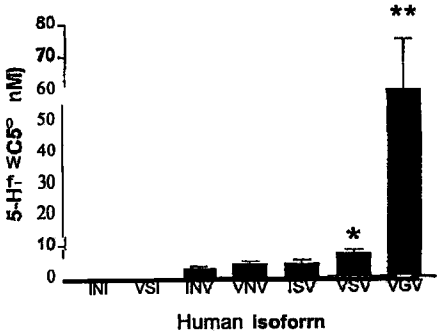

FIG. 2. 5-HT potency to activate phosphoinositide hydrolysis in NIH.3T3 fibroblasts transiently expressed with human edited 5.HT$_{2C}$Rs. The human 5-HT$_{2C\text{-}INI}$, 5-HT$_{2C\text{-}VSI}$, 5-HT$_{2C\text{-}INV}$, 5-HT$_{2C\text{-}VNV}$, 5-HT$_{2C\text{-}ISV}$, 5-HT$_{2C\text{-}VSV}$, and 5-HT$_{2C\text{-}VGV}$ isoforms were transiently expressed in NIH-3T3 cells, and the EC$_{50}$ of 5-HT for activation of phosphoinositide hydrolysis was determined as described under "Experimental Procedures." Values represent the mean ± S.E. of5-11 separate determinations for each isoform. B_{max} values, determined by binding analyses with a single 8 nM concentration of [^{3}H]mesulergine, were as follows: 5-HT2C_INI, 900 = 259 fmol/mg; 5-HT2C_VSI' 1399 ± 626 fmol/mg; 5-HT$_{2C\text{-}ISV}$ 627 ± 133 fmol/mg; 5-HT$_{2C\text{-}VNV}$, 1617 ± 339 (mol/mg; 5-HT$_{2C\text{-}INV}$, 732 ± 280 fmoVmg; 5-HT$_{2C\text{-}VSV}$, 1669 ± 384 fmol/mg; and 5-HT$_{2C\text{-}VGV}$, 422 ± 36 fmol/mg. These values were determined not to be statistically different based upon one-way analysis of variance. *, the EC$_{50}$ value of 5-HT was significantly different from5-HT2C_INI, 5-HT$_{2C\text{-}VSI}$, and 5-HT$_{2C\text{-}INV}$ as determined by individual unpaired Student's t tests (p = 0.005, 0.0197, and 0.0347, respectively). **, the EC$_{50}$ value of 5-HT was significantly different from all other examined isoforms as determined by individual unpaired Student's t tests (p < 0.015 in all cases).

the 5-HT2C_VSV or 5-HT$_{2C\text{-}VGV}$ isofonns as compared with the 5-HT2C_INI receptor (Fig. *5A)*. The 5-HT2C_VGV receptor-expressing cells, however, did not exhibit the substantial EC$_{50}$ shift seen in the transient experiments. It is possible that the higher receptor expression in the 5-HT2C_VGV receptor line resulted in a significant receptor reserve for the 5-HT response, causing the EC$_{50}$ value to appear inappropriately low. To address this possibility, 5-HT2C_VGV receptor cells were treated with the alkylating agent phenoxybenzamine (25). This treatment inactivated approximately 60% ofthe 5-HT$_{2C\text{-}VGV}$ receptors, resulting in a statistically equivalent density between the three cell lines, thereby allowing a more direct comparison of EC$_{50}$ values. Subsequent stimulation of this partially inactivated population with 5-HT revealed that stably expressed 5-HT2C_VGV receptors now exhibited a decreased maximum response (20% of untreated cells) and a 29-fold lower EC$_{50}$ for 5-HT when compared with the 5-HT$_{2C_INI}$ isoform (Fig. *3A)*. This potency shift agrees closely with the 26-fold difference determined in transient transfection analyses (Fig. 2)_

Stimulation with other 5-HT$_{2C}$R agonists revealed similar discrepancies in EC$_{50}$ values between different edited isoforms. {::!::}-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane (DOI), a hallucinogenic agonist with structural similarity to amphetamine, was 9- and 43-fold less potent at the 5-HT$_{2C\text{-}VSV}$ and 5-HT2C_VGV receptors, respectively, when compared with its ability to activate signaling of the 5-HT$_{2C_IN1}$ isoform (Fig. *3B)*. *N,N*-dimethyltryptamine (DMTl, a hallucinogenic indoleamine, was 40-fold less potent at the 5-HTzc_vsv receptor and 91-fold less potent at the 5-IIT2C_VGV variant compared with the response generated by interaction of this ligand with the 5-HT$_{2C\text{-}INI}$ receptor (Fig. *3C)*.

Two Agonist Affinity States Are Observable Only for the 5-HT$_{2C\text{-}INI}$ Isoform-According to current theory of receptor/G protein interaction, the coupling of receptor with G protein results in an increase in the affinity ofagonists for the receptor. Competition binding analyses were performed to evaluate differences in agonist affinity states for the 5-HT$_{2C_IN1}$ and 5-HT$_{2C\text{-}VGV}$ receptors (Fig. 4). Competition binding regularly revealed a low and high affinity state upon interaction of5-HT with the 5-HTzc_INI receptor (Fig. *4AJ;* competition binding curves could never be fit to a two-site model for 5-HT2C_VGV receptors (Fig. 4B; Table Il. Data with the 5-HTzc_vsv receptor was variable, sometimes revealing two sites, but more {lften best fit by a one-site model (data not shown). The 5-HT high affinity population of 5-HT2C-INI receptors could be shifted to low afiinity when binding analyses were performed in the presence of 100 μM Gpp(NH)p, while the curves for the edited 5-HT2C_VGV receptor remained unchanged. In the presence of Gpp(NH)p, significantly higher affinities were consistently observed for agonists when the 5-HT$_{2C\text{-}INI}$ receptor was compared with the 5-HT$_{2C_V}$(w variant (1'ablc 1). This was not the case, however, when competition analyses were performed with mianserin, an inverse agonist at the 5-HT$_{2C}$R and a ligand that is predicted to preferentially interact with uncoupled receptors.

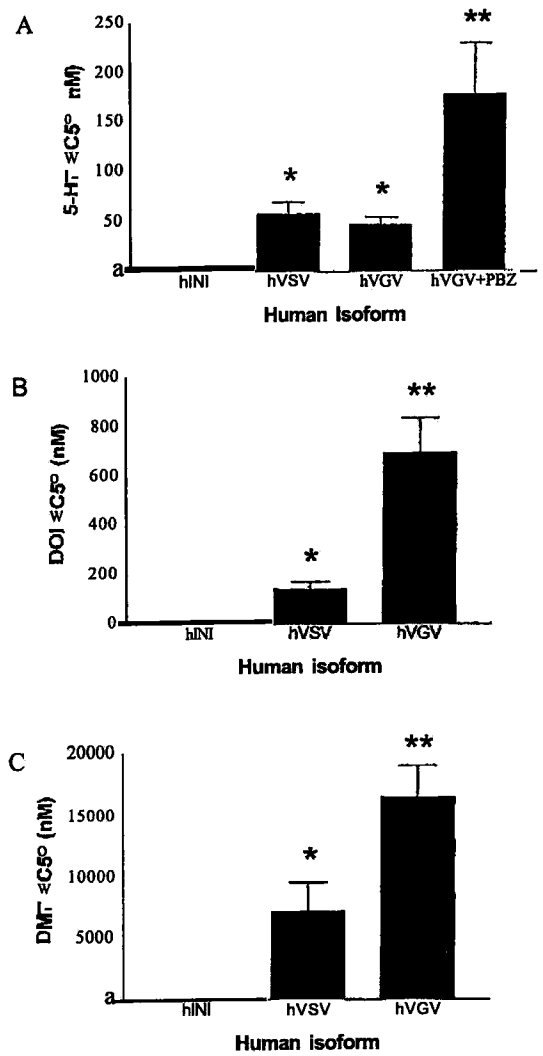

FIG. 3. Differential responses of agonists to activate phosphoinositide hydrolysis in human edited 5.HT$_{2C}$R stable cell lines. The EC_{50} for activation of phosphoinositide hydrolysis by 5-HT *(A)*, DOI *(E)*, and DMT *(C)* in NIH-3T3cells stably expressing 5-HT$_{2c\text{-}INI}$' 5-HT2C_VSV' and 5-HT$_{2C\text{-}VGV}$ receptors is shown. Values represent the mean::': S.E. of 3–16 independent determinations performed in triplicate for each drug. Receptor densities (B_{max}) were as follows: 5-HT$_{2C_INI}$' 258 ± 87 fmol/mg; 5-HT2C_VSV' 173 ± 70 fmol/mg; and5-HTzc_vcv, 1054 ± 260 fmoVmg (n = 4-6). Inactivation of 5-HTzc_vcv receptor-expressing cells with 3.2 μM phenoxybenzamine resulted in a decrease in receptor density to 305 = 141 fmolJmg (n = 3). *A*, *, these values were significantly different from 5-IIT$_{2C\text{-}INI}$ (p = 0.0141 for 5-HTzc_vsv, p = .0059 for 5-HTZC_VCY). **, this value was significantly different from 5-HTzc.vcv alone (p < 0.0001). 5-HT$_{2C\text{-}VSV}$ and 5-HTZC_YGV were not significantly different from each other (p = 0.4550). *B*, *, a significant difference from 5-HT$_{2C\text{-}INI}$ (p = 0.0160). **, a significant difference from 5-HTzc_INI (p = 0.0201) and 5-HT2C_VSV (p = 0.0476). C, *, a significant difference from 5-HT2C_INI (p = 0.0388). **, a significant difference from 5-HT$_{2C_INI}$ (p = 0.0006) and 5-HT$_{2C\text{-}VSV}$ (P = 0.0444). All statistical analyses were performed using individual unpaired Student's t tests.

These results again support the hypothesis that the 5-HT$_{2C\text{-}INI}$ receptor couples more efficiently to G proteins, even in situations where no agonist is present to induce the coupled state.

Constitutive Activity Differences between Human 5-HT$_{2C}$R Isofarms-Rat 5-H1'$_{2C}$Rs have been shown to exhibit constitutive activity (22, 23), defmed as the ability of a receptor to stimulate downstream signaling events in the absence of agonist occupation (reviewed in Ref. 26). The binding studies described previously suggested that the edited receptors might exhibit differential abilities to stimulate basal levels of inositol phosphate hydrolysis. Studies of constitutive receptor activity were performed in COS-7 cells, where transient expression gave reproducibly high levels of receptor expression, which is needed to accurately evaluate agonist-independent activity. An analysis of transient transfection experiments in which the nonedited and edited receptors were expressed at similar densities indicated that the 5-HT$_{2C\text{-}INI}$ isoform elicited 5-fold greater levels of basal [^{3}H]inositol monophosphate generation compared with the 5-HT2C_VGV receptor (Fig. 5). The addition of 1 μM methysergide, a neutral antagonist, blocked 5-HT-stimulated inositol phosphate formation but had no effect on the basal activity of the 5-H1'2c_'N' receptor (data not shown). The disparity in basal activity mirrors the differences in "precoupiing" ability reflected in the competition binding experiments, again suggesting that the 5-H1'2C_INI receptor interacts more efficiently with the G protein linked to phospholipase C. Importantly, these analyses also revealed that similar maximal responses were obtained for both receptor isoforms (Fig. 5), indicating that the primary effect of editing may be to alter agonist-independent activity rather than affecting the intrinsic ability of receptor isoforms to promote G protein coupling.

DISCUSSION

RNA editing is a post-transcriptional process that contributes to molecular diversity within cells. 5-H1'$_{2C}$R RNA transcripts undergo adenosine-to-inosine RNA editing events resulting in the generation of distinct amino acids within the second intracellular loop of the protein (7), a region known to be important for G protein coupling (9-16). Editing within rat 5-H1'2cR RNA is mediated by the coordinated actions of a family of adenosine deaminases termed ADARs (adenosine geaminases that act on RNA; Refs. 7 and 27). These editing events are conserved among rodent and human species (8), suggesting that they serve an important role in receptor function.

Editing in the human brain creates a different complement of receptor isoforms due to an increase in the percentage of isoforms edited at position 158, most notably the 5-H1'2c_vSV and 5-HT2C_VGV isoforms. Transient transfection of the principal human isoforms revealed that 5-H1' exhibited the lowest potency at the novel 5-HT2C_VGV receptor. The 5-HT$_{2C\text{-}VSV}$ receptor was the only other isoform to show a significant difference in 5-HT potency relative to the nonedited isoform. In cell lines stably expressing the 5-HT$_{2C\text{-}INI}$, 5-HT$_{2C\text{-}VSV}$, or 5-HT$_{2C\text{-}}$vav receptors, agonists exhibited a decreased potency when interacting with the edited receptor isoforms with the effect again being most dramatic upon interaction with the 5-HT2C_VGV receptor. The potency difference between 5-HT$_{2C\text{-}VGV}$ and 5-HT$_{2C_INJ}$ receptors was augmented when a majority of the 5-H1'2C_vGV receptors were inactivated \vith phenoxybenzamine, indicating that the observed potency phenotypes are not due to variations in receptor reserve between different receptor populations. Instead, these results are consistent with the interpretation that certain edited receptors exhibit a reduced G protein coupling capacity.

As a first step in defining the mechanism responsible for the reduced potency of agonists at edited 5-HT$_{2C}$Rs, competition binding profiles were examined to exploit the differential affinities of an agonist for G protein-coupled *versus* G protein-uncoupled receptor populations. The 5-HT competition binding curves for 5-HT$_{2c\text{-}rnl}$ receptors were shallow and best fit by a

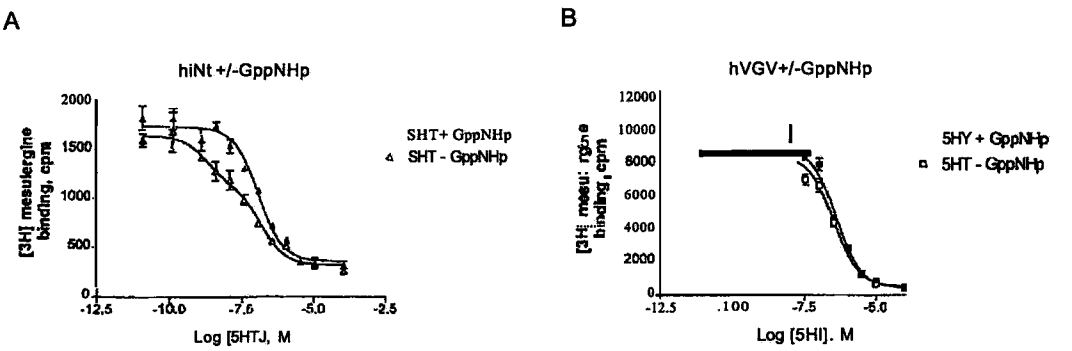

FIG. 4. 5-HT affinity for human edited 5-HT$_{2C}$Rs and modulation by the GTP analogue Gpp(NII)p. Competition binding analyses are shown for 5-HT$_{2C\text{-}INI}$ *(A)* and 5-HT$_2$c-vcv receptor cell lines *(B)* in the presence *(closed symbols)* and absence *(open symbols)* of 100 μM Gpp(NH)p. Increasing concentratiolls of 5-HT woro incubated with [^{3}H]mesulergine as described under "Experimental Procedures." Data were fitted to both one- and two-site models using GraphPad Prism software. The mean K_i values for 5-HT, determined by the method of Cheng and Prusoff, for 5-HT$_{2C\text{-}INI}$ were 0.68 ± 0.13 nM (high affinity) and 85.7 ± 17.2 nM (low affinity) in the absence of Gpp(NH)p and 57.4 ± 12.0 nM in the presence of 100 μM Gpp(NH)p. For 5-HT2C_YGV' the mean K_i values for 5-HT were 167.0 ± 16.1 nM in the absence of Gpp(NH)p and 302.4 ± 46.4 nM in the presence ofIOO μM Gpp(NH)p, respectively. K_d values for (3HJmesulergine, determined in the presence ofIOO μM Gpp(NH)p, were as follows: 0.99 ± 0.07 for 5-HT$_{2C\text{-}INI}$ and 1.09 ± 0.21 for 5-HT$_{2C\text{-}VGV}$ (p = .60n Data represent the mean ± S.E. oUouror five independent determinations performed in duplicate.

TABLE I

Relative affinities of agonists (or 5-HT$_{2C\text{-}INI}$ and 5-11T2c.vcv receptors

The K_i values of 5-HT, DOL, DMT, LSD, and mianserin were determined by competition for 1 nM [^{3}H]mesulergine binding in the absence or presence of 100 μM Gpp(NHlp. Data were fit to one- and two-site models based on the best fit determined by using GraphPad Prism software. Values represent the mean ± S.E. of 3-6 independent analyses performed in duplicate. K_i values were calculated using the method of Cheng and Prusoff (24) using the mesulergine K_d values, determined in the presence of 100 μM Gpp(NII)p, of 0.99 ± 0.07 for 5-HT$_{2C\text{-}INI}$ and 1.09 ± 0.21 for 5-HT$_{2C}$-vcv. Data were statistically analyzed by unpaired Student's *t* test.

Agonist	HumanINI	Human VGV	*p*
5-HT without GppNHp			
High	0.68 ± 0.13		
Low	85.7 ± 17.2	167 ± 16.1	0.0007
R" (%)	46.2 ± 5.7		
5-HT with GppNHp	57.4 ± 12.0	302.4 ± 46.4	0.0118
DOI with GppNHp	41.0 ± 3.7	70.4 ± 9.0	0.0240
DMT with GppNHp	382.3 ± 47.0	1235 ± 122.2	0.0006
LSD with GppNHp	4.35 ± 0.94	12.9 ± 1.4	0.0028
Mianserin with GppNHp	6.98 ± 1.16	3.59 ± 0.86	0.0574

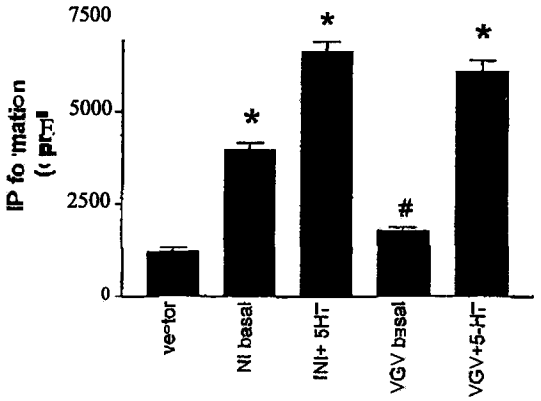

FIG. 5. Constitutive activity of transiently expressed 5-HT$_{2C\text{-}INI}$ and 5-HT$_{2C\text{-}VGV}$ receptors. cDNAs for edited receptors were transiently expressed in COS-7 cells, and basal [^{3}H]inositol monophosphate formation was measured after a 35-min incubation. #, $p < 0.05$ *versus* vector alone; *, $p < 0.01$ compared with vector and the 5-HT$_{2C\text{-}VGV}$ receptor cell line. The densities of the receptors, estimated using a single concentration of [^{3}H]mesulergine (0.5 nM), were 2.7 ± 0.2 pmollmg for 5-HT2C_1Nl and 2.9 ± 0.2 pmoJJmg protein for 5-HT2C_VGV.

two-site competitive binding model. The high affinity state of 5-HT, representing approximately 50% of the binding, was eliminated by the addition of the GTP analog, Gpp(NH)p. In contrast, the 5-HT competition binding curves with 5-HT2C_VGV receptors were best fit by a one-site competitive binding model, and the affinity approximated the low affinity site found with the 5-HT2C_INJ isoform_ For the 5-HT$_{2C\text{-}VSV}$ receptor, the curves sometimes fit two sites (data not shown), but most often the one-site competitive binding model gave the best fit. These results demonstrate that, compared with the5-HT2C_VSV and 5-HT2C_vGV receptor populations, a relatively large proportion of 5-HT2c_rNI receptors exists in a G protein coupled state, providing the first direct evidence that RNA editing generates 5-HT$_{2C}$Rs that differ in the efficiency ofcoupling to G proteins. Thus, there appear to be graded states ofprecoupling ability for the edited 5-HT$_{2C}$Rs, with 5-HT2c_rNr receptor being most efficacious at G protein coupling, the5-HT2c_vsv variant being intermediate, and 5-HT2C_VGV receptors existing predominantly in the uncoupled. state.

The ternary complex model of receptoriG protein interaction has recently lUldergone revision to accommodate the finding that some receptors have the ability to induce effective G protein coupling in the absence of an agonist, termed constitutive activity (28). The model predicts that receptors have the capacity to spontaneously isomerize from an inactive conformation, termed R, to an active state, R*, with the R* version of the receptor having the ability to interact with G proteins. In experimental paradigms studying the properties of constitutively active mutant receptors, it has been observed that these receptors exhibit both a higher level of basal activity and a greater potency for agonists (28, 29). 8illoo the nonedited 5-HT$_{2C}$R exhibits constitutive activity (22, 30), we considered the possibility that the agonist potency differences generated by RNA editing may reflect differential abilities to isomerize to the R* state, with the 5-HT2C.INI receptor existing more readily in the R* form compared with the edited 5-HT2C_VSV and 5-HT2C_VGV isoforms. To evaluate alterations in constitutive activity, the basal activity of the nonedited 5-HT$_{2c\text{-}INI}$ receptor was compared with the fully edited 5-HT2C_VGV isoform, which exhibited the greatest shift in 5-HT potency. In COS-7 cells transiently expressing 5-HT$_{2C\text{-}INI}$ receptors, basal phospho-

inositide hydrolysis was substantially increased in comparison with mock-transfected cells. In contrast, the 5-HT2C_VGV receptor isoform supported only a modest degree of basal [3H] inositol phosphate formation, about 20% of that produced by 5-HT2C-INI receptors expressed at a comparable density. These results indicate that the fully edited 5-HT2C_VGV receptor has a much lower intrinsic ability to support agonist-independent phosphoinositide hydrolysis than does the nonedited receptor isoform. Two alternative possibilities could explain the differences in 5-HT$_{2C\text{-}INI}$ and 5-HT$_{2C\text{-}VGV}$ receptor constitutive activity. One hypothesis is that large differences in the fraction of receptors residing in the R* conformation might underlie the distinction in constitutive activities. An alternative explanation is that both receptor isoforms exhibit the same ability to convert to R*, but the combination of amino acids within the 5-HTzc_INI receptor promotes more efficient interaction with the G protein, due to either the favorable conformation of the second intracellular loop or actual contact of the nonedited amino acids with the G protein. In experiments designed to test the latter hypothesis, we found that 5-RT treatment elicited an increase in [^{3}H]inositol phosphate formation of the same magnitude in cells expressing equal densities of 5-HT$_{2C\text{-}INI}$ or 5-HT2C_VGV receptors, strongly suggesting that the two receptor isoforms have equivalent ability to couple to G proteins when bound by agonists. Thus, these results indicate that 5-HT$_{2C\text{-}INI}$ receptors have a greater propensity to spontaneously isomerize into or maintain the active R* conformation than 5-HTzc_vGV receptors. Thus, we propose that one consequence of RNA editing of the 5-HT$_{2C}$R is to silence its constitutive activity, thus increasing the signal:noise ratio at sites where editing efficiency is high.

The extended ternary complex model predicts that receptor isofonns with high levels of constitutive activity would exhibit higher affinity for agonists, even in the absence of G protein coupling (28, 29). As a first step at evaluating agonist affinities in the absence of G proteins, we compared the binding affinities in the presence of Gpp(NH)p, assuming that this reflects the affinity of the G protein-uncoupled receptor. The affinity of 5-HT for the 5-HT2C_VGV receptor was 5-fold lower than for the 5-HT$_{2C\text{-}INI}$ isoforro; significant differences were also found for the other agonists tested but not for the inverse agonist mianserin. These observations suggest that some structural perturbation allows the edited receptors to assume an inactive receptor conformation with lower affmity for agonists. Studies of agonist affmities using purified receptor protein are needed to definitively show that the agonist affinity differences do not depend on G protein interactions.

Recent structure-activity studies of other G protein-coupled receptors point to the importance of amino acid residues in positions analogous to the edited sites in the dynamics of receptor/G protein interactions. Amino acid residues that reside at positions analogous to IlcIVal^{156} and Ile/Val^{160} in other G protein-coupled receptors are important in terms of general G protein coupling ability and receptor regulation (10, 12, 16l. For example, within the m1 and m3 muscarinic receptors and the β2 adrenergic receptors, the amino acid at IleNal^{160} must be bulky and hydrophobic to induce a productive G protein interaction (10), consistent with our evidence that editing at position 160 contributes to a low efficiency of G protein coupling. Molecular modeling of the gonadotropin-releasing hormone receptor has shown that the Ile corresponding to the 5-HT$_{2C}$R IleNal^{156} is critical for forming a "cage" around the arginine of the highly conserved DRY sequence in the second intracellular loop (16). It was proposed that the "arginine cage" stabilizes interaction with the adjacent aspartate residue, thereby enabling the receptor to remain in an active state.

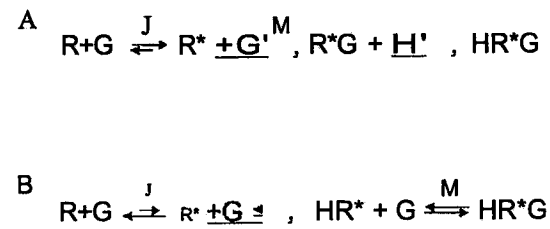

FIG. 6. Model of regulation of 5-HT$_{2C}$Rs by RNA editing. 5-HT2C_INI (A) and 5-HT2C_VGV (B) receptors exhibit differential abilities to isomerize to the productive R* state. Conversion to this coupling-competent receptor version is represented for 5-HT$_{2C\text{-}INI}$ by the large *J* constant and *long forward arrow*. 5-HT$_{2C\text{-}VGV}$ receptors can convert to the productive R* state but only when agonist is present.

Alteration of the Ile/Val^{156} position of the 5-HT$_{2C}$ receptor in the context of other edited amino acids might produce different conformations of this critical arginine, allowing some edited isoforms to couple more efficiently than others. The expression of more than 12 predominant isoforms in the human brain further suggests that unique degrees of coupling ability might be produced with each distinct edited receptor.

The consequences of RNA editing within 5-HT$_{2C}$R RNA are summarized in the model presented in Fig. 6, with *A* representing the 5-HT2C_INI receptor and *B* representing the 5-HT2C_VGV receptor isoform. In this model, the 5-HT2C_INI receptor has a much greater capacity to isomerize to the active R* conformation (represented in *boldface type* and described by the constant *J*). The R* form of the receptor interacts efficiently with G proteins in the absence of agonist *(m;* upon agonist binding, the response is enhanced. For edited 5-HT2C-VGV receptors, however, the *J* constant is much smaller, reflecting a decreased spontaneous ability of these receptors to convert to the coupling-competent state. It is only upon binding of agonist that these receptors achieve the ability to significantly convert to a form able to elicit efficient G protein interaction. Our current working hypothesis is that the edited receptor has a lower value for the J constant, while the M constant, an index of the intrinsic ability of an isoform to couple to G proteins, remains relatively constant.

In summary, the current results demonstrate that the 5-HT$_{2C\text{-}INI}$ receptor isoform exhibits a greater level of constitutive activity than does the edited 5-HT$_{2C\text{-}VGV}$ receptor isoform and therefore possesses a greater propensity to spontaneously isomerize to the R* state. The differential degrees of constitutive activity and the accompanying secondary alterations in agonist potency and G protein coupling could have important implications for the physiological effects of 5-HT. Brain regions that contain the nonedited receptor would be more sensitive to 5-HT that may be tonically released at distinct sites, possibly generating considerable noise in the system. In addition, the magnitude of signal produced by firing of presynaptic serotonergic terminals may be reduced because of a high basal tone at the constitutively active 5-HT2C_INI receptor. Region-specific generation of edited isoforms, coupled with the possibility that individuals have different editing patterns, suggests that the repertoire of expressed edited 5-HT$_{2C}$Rs might determine the response to endogenous serotonin as well as control the signal:noise ratio at central serotonergic synapses.

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REFERENCES

1. Sommer. B., Kohler, M., Sp.engel. fl., and Seeburg, P. H. (1991) *Cell* 67, 11–19
2. Hollmann, M. Hartley. M., and Heinemann, S. (1991) *Science* 252, 851-854

3 Verdoorn, T. A., Burnashev, N., Monyer, H., Seeburg, P. H., and Sakmann, B. *(1991) Science* 252,1715-1718
4 Bruse, R., Zimmermann. F., Koh, D.-S., Feldmeyer, D., Gass, P., Seeburg, P. H., and Sprengel, R. (1995) *Science* 270, 1677–1680
5. Hoyer, D., Clarke, D. E., Fozard, J. R, Hartig, P. R., Martin, G. R, Mylecharane, E. J., Saxena, P. R., and Humphrey, P. P. (1994) *Pharmacol. Rev. 46,* 157–203
6. Sanders-Bush, E., and Canton, H. (1996) in *Psychopharmacology: Fourth Generation of Progress* (Bloom, F. E., and Kupfer. D. J., eds) pp. 431-441, Raven Press, NY
7. Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H , Sanders-Bush, E., and Emeson, R. B. (1997) *Nature* 387, 303-308
8 Niswender, C. M., Sanders-Bush, E., and Ellesun, R. B. (1998) *Ann. N. Y. Acad. Sci.*, 861, 38–48
9 Weng, S. K, Parker, E. M., and Ross, E. M. (990) *J. Biol Chem.* 265, 62J9-6224
10. Mora, 0., Lameh J., Högger, P., and Sadée, W. (1993) *J. Biol. Chem. 268,* 22273–22276
11. Pin, J. P., Joly, C., Heinemann, S. F., and Bockaert, J. (1994) *EMRO J. 13,* 342–348
12. *Arora,* K K., Sakai, A., and Catt, K (19951 J, *Biol. Chem.* 270, 22820–22825
13 Rlin, N" Yun, J., and Wess, J. (1995) *J. Biol. Chem.* 270, 17741-17748
14 Arora, K K, Cheng, Z, and Catt, K. (1997i *Mol. Endocrinol.* 11, 1203–1212
15. Vacrell, S., Ishii, M., Chen, M., Wang, L., Tram, T., and Coughlin, S. (J997) *J. Biol. Chem.* 272, 6898-6902
16. Ballesteros, J., Kitanovic, S., Guarnieri, F., Davies, P., Fromme, *B.,* Konvidm, K., Chi, L., Millar, R. P., Davidson, J. S., Weinstein, H., and Sealfon, S. C. (1998) *J. Riol. Chem.* 273, 10445-10453
17. Saltzman, A. G., Morse, n., Whitman, M, M., Ivanshchenko, Y., Yaye, M., and Felder, S. (1991J *Biochem. Riophys. Res. Commun,* 181, 1469-1478
18. Xie, E., Zhu, L., Zhao. L., and Chang, L. S (1996) *Genomics* 35, 551-561
19. Ausubel, F. M., Brent, R., Kingston, R. Moore, D., Seidman, J., Smith, J., and Struh], K. (eds) (1989\ *Current Protocols in Molecular Biology.* John Wiley & Sons, Inc., New York
20. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci.* U. S. *A.* 82,488-492
21. Herrick-Davis, K. Egan, C., and Teitler, M. (1997) *J. Neurochem. 69,* 1138-1144
22. Barker, E. L., Westphal, R. S., Schmidt, D., and Sanders-Bush, E. (1994) *J. Biol. Chem.* 269, 11687_11690
23 Westphal, R. S., and Sanders-Bush, E. (1994) *Mol. Pharmacol.* 46,937-942
24. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* 22,3099-3108
25. Furchgott, R. F., and Bursztyn, P. (1967) *Ann. N. Y. Acad. Sci.* 144, 882–893
26. Lefkowitz, R. J., Cotecchia, S., Samama, P., and Costa, T. (1993) *Trends Pharmacol. Sci.* 14,303-306
27. Bass, B. L. (1998) in *The RNA World* (Gesteland, R. F., and Atkins, J. F., eds) pp. 383–419, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Samama, P., Cotecchia, S., Costa T., and Lefkowitz R. J. (998) *J Biol. Chem.* 268, 4625–4636
29. Perez, D. Ill., Hwa, J., Gaivin, R., Mathur, M., Brown, F., and Graham, R. M. (996) *Mol. Pharmacol.* 49, 112-122
30. Hartman, J. L., IV, and Northup, J. K. (19961J Biol. *Chem.* 271, 22591–22597

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A Rapid New Assay to Detect RNA Editing Reveals Antipsychotic-Induced Changes in Serotonin-2C Transcripts

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ABSTRACT

We report the development of a new assay as an alternative to direct DNA sequencing to measure RNA-edited variation in tissue. The new assay has been validated and is accurate, cheaper, more rapid, and less labor-intensive than DNA sequencing. We also outline the statistical modeling required for analyses of the hierarchical, clustered RNA-editing data generated in these studies. Using the new technique, we analyzed the effects of long-term antipsychotic medication on serotonin-2C receptor (5-HT_{2C}R) RNA editing in rat brain. Our hypothesis that a drug with high affinity for 5-HT2CR, such as c1ozapine, would alter its RNA-editing profile was not confirmed. Whereas haloperidol, a typical antipsychotic drug that is primarily a dopamine receptor antagonist, reduced 5-HT2C VNV isoform frequency and the level *of* RNA editing at the D site, risperidone and not the prototype atypical antipsychotic drug c10zapine increased the frequency of 5-HT_{2C} VNV and D-site editing. Our data emphasize that caution is required in the interpretation of RNA-editing data in studies of psychiatric disorders, because these studies usually include subjects who received long-term exposure to medication. This newly established method will facilitate high-throughput investigations of RNA editing in disease pathology and in the pharmacological activity of drugs.

The revelation that the human genome comprises between 30,000 and 40,000 protein-coding genes was unexpected, because such few genes are unlikely to explain the functional diversity between humans and less complex organisms. These interspecies differences could arise from post-transcriptional processes such as RNA editing and alternative splicing, which anow a single gene to generate several protein variants. The current study focuses on RNA editing. The recent detection of 1637 potential new substrates for RNA editing (Levanon et al., 2004) indicates that it is likely to be more widespread in the transcriptome than originally believed. RNA editing occurs in the pre-mRNA and has many forms, including insertion, deletion, and the conversion of cytidine to uridine or adenine to inosine. In the latter case, editing of specific adenine r-esidues involves their hydrolytic deamination by a double-stranded RNA adenosine deaminase (ADAR) enzyme. Because inosine base pairs with cytidine in transfer RNA, it is predicted to be translated as guanidine by the ribosome. These changes alter the codon and often the amino acid sequence of the protein, usually with functional-consequences (Gatt, 2003),

Because of their relatively recent discovery, substrates of RNA editing have not been extensively characterized. Adenine-to-inosine RNA editing in mammalian brain has been identified in ionotropic receptors (such as the GluR2 subunit of the α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor) and in the serotonin f5-HT)-2C receptor (R), which is coupled to GTP-binding protein. Discrepancies between genomic DNA and cDNA sequences led to the discovery of nucleotide changes caused by the activity of ADAR enzymes. In the fully edited '5-HT_{2C}R, three amino acid codons in the pre-mRNA are changed so that the sequence coding for IRNPI becomes VRGPV (Fig. 1) (Burns et al., 1997),

RNA editing produces many coexisting, functionally dis-

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ABBREVIATIONS: 5-HT_{2C}R, 5-HT_{2C} receptor; ADAR, adenosine deaminase acting on RNA; peR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; ICC, intraclass correlation coefficient; GEE, generalized estimating equation; OR, odds ratio; bp, base pair; GI, confidence interval.

tinct variants of 5-HT_{2C}R. RNA extracted from tissue expressing the 5-HT_{2C} gene is a heterogeneous mixture of up to 32 different 5-HT_{2C} mRNA transcripts. These are predicted to encode 24 protein isoforms and have been detected in both rodent and human brain, with regional differences in their frequencies (Burns et al., 1997; Niswender et al., *2001).* Pharmacological evidence shows that 5-HT_{2C} RNA editing reduces constitutive activity and G-protein activation in response to agonist stimulation (Niswender et al., 1999). Therefore, the RNA editing process represents a regulatory mechanism by which cells can modulate their response to environmental stimuli by altering the efficacy and specificity of G-protein to receptor interactions. It is possible that disruption of this process could have pathophysiological effects, but this has not been extensively explored because RNA editing is difficult to detect and quantify.

Limited data indicate that altered RNA editing is associated with a variety of disease processes. Most notably, reduced RNA editing of GluR2 has been found in spinal motor neurons of patients with amylotrophic lateral sclerosis (Kawahara et al., 2004). Furthermore, altered levels of RNA-editing enzymes have been detected in inflamed tissues (Yang et al., 2003) and in malignant gliomas (Maas et al., 2001), whereas studies of psychiatric disease have revealed altered 5-HT_{2C} RNA-editing patterns in schizophrenia (Sodhi et al., 2001) and in suicide (Niswender et al., 2001; Gurevich et al., 2002). However, there are conflicting data (Dracheva et al., 2003), and whether the observed changes in subjects treated with antidepressants are caused by drug treatment is unresolved (Gurevich et al., 2002). More extensive investigations are needed to clarify these findings. Until now, this has been a daunting task, because the complete assessment of RNA editing has involved extensive DNA purification and sequencing, impeding replication studies in the large number of subjects needed for optimum statistical power (see *Results).* Therefore a high-throughput RNA-editing detection method would be invaluable.

Herein, we present a newly developed assay for RNA editing modeled on one of the more complex substrates, the multiply edited 5-HT_{2C} pre-mRNA. The new RNA-editing detection assay is as accurate and informative as DNA sequencing. It is much cheaper and can be adapted for high-throughput protocols and for other RNA-edited substrates. Using this assay, we tested the effects of antipsychotic treatments on 5-HT2c mRNA editing. Furthermore, we clarify the hierarchical sampling design of RNA-editing data sets, investigate the impact of clustering on statistical power analyses, and guide statistical modeling choices appropriate for future studies of RNA editing.

Materials and Methods

Preparation of 5-HT_{2C} cDNA Template. Messenger RNA (2 μg) was extracted by standard methods and pretreated at 37°C for 30 min with 1 unit of RQ-1 RNase-free DNase and 10 units of RNasin RNase inhibitor (both from Promega, Madison, WI), followed by 6 min at 70°C. Reverse transcription was carried out using 200 units of Moloney murine leukemia virus reverse transcriptase with 10 units of RNase inhibitor (Promega), 30 ng of poly(dT) oligonucleotides (Oswel, Southampton, UK), buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol), and a 0.5 mM concentration of each dNTP (Promega). The reaction mixture was incubated at 42°C for 1 h followed by 72°C for 10 min. The cDNA produced was

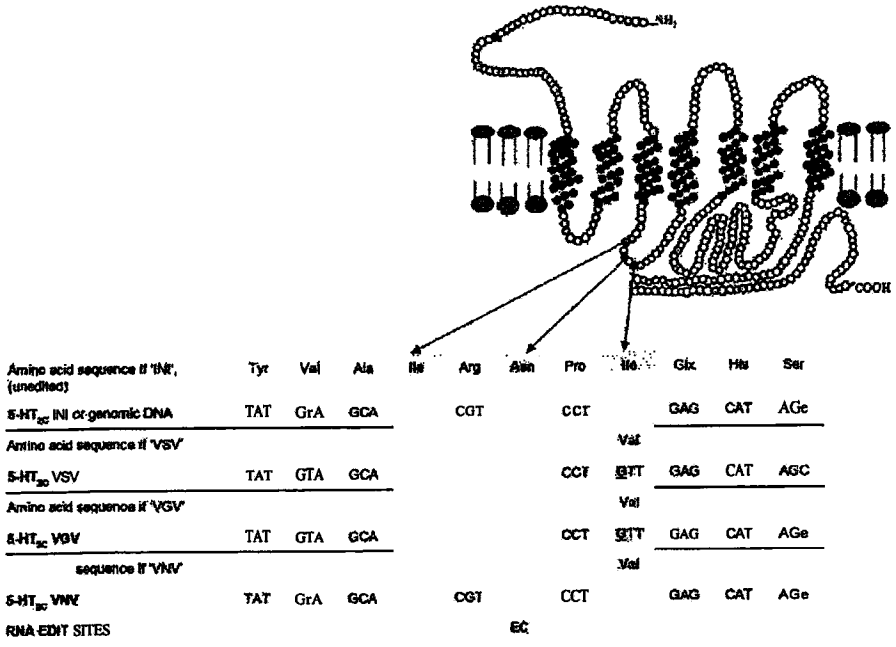

Fig. 1. Sequence changes observed in the 5-HT_{2C} RNA edited region. The sites of A to I editing, detected as "A" to "G" changes, are indicated as underlined Gs. The top sequence represents the genomic DNA sequence, which is also detected in the cDNA when RNA editing has not occurred (i.e., in the 5-HT_{2C} INI unedited isoform). The sequence of one of the frequently edited isoforms, 5-HT_{2C} VSV, has "A" to "G" changes at sites A-D, which alter three amino acids. The 5-HT_{2C} VAV fully edited isoform has "A" to "G" changes at sites A-E, leading to three altered amino acids. 5-HT_{2C} VNV, 5-HT_{2C} VSV, and 5-HT_{2C} VINI isoforms were found to be frequent in this study.

diluted with nine parts of sterile deionized water. The subsequent PCR amplification of the 5-HT_{2C} RNA-edited region was performed using oligonucleotide primers designed to span the rat exon 3/intron 3 boundary to prevent the amplification of residual contaminant genomic DNA. This peR reaction included template eDNA (90% dilution), 1 mM concentration of each primer MSF (5'-TTTCAACT-GCGTCCATCATGCACCT-3') and MSR (5'-AACGAAGTTGGGGT-CATTGAGCAC-3'), 250 mM concentration each of dATP, deTP, dGTP, and dTTP (dNTPs), 2 units of DNA polymerase and recommended buffer (Qiagen, Valencia, CA) in a total volume of 20 μl. Samples were amplified for 27 cycles (in the linear phase of amplification). Cycling conditions comprised 1 cycle at 96°C for 5 min followed by 27 cycles at 96°C for 15 s, 50°C for 30 s, and 72°C for 30 s, and finally 1 cycle of 72°C for 5 min in a thermal cycler (Peltier; MJ Research, Watertown, MAI, resulting in a 236-bp product. DNA fragments were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide (50 ng/ml), excised from the gel, and purified using Qiaex II (Qiagen).

Characterization of RNA Edited Transcripts. The gel-purified DNA was ligated and doned using the pGEM-T system II (Promega). Randomly picked colonies were either characterized using direct sequencing or were identified using the new pyrosequencing method (Fig. 2). The DNA sequencing protocol required overnight subcloning, and the DNA was purified and sequenced according to the automated ABI protocol using an ABI377 sequencer (Applied Biosystems, Warrington, UK).

In contrast, with the new pyrosequencing protocol, the liquid culture and DNA purification steps were not necessary. Instead, bacteria from each colony were directly inoculated in a PCR reaction mixture containing 0.2 μM sense oligonucleotide primer (5'-ATATCGCTGGATCGGTATGTAG-3') and 0.2 μM antisense biotinylated primer (5'-BIOTIN CGAATTGAAACGGCTATGCT-3'), Ix Taq Gold buffer with 1.5 mM $MgCl_2$ (PerkinElmer Life and Analytical Sciences, Boston, MAI, 0.75 units of Taq Gold (PerkinElmer), and 0.24 mM dNTPs. Samples were heated to 96°C for 5 min and then amplified for 45 cycles each consisting of 20 s at 96°C, 30 s at 58°C, and 20 s at 72°C in a thermal cycler (Peltier; MJ Research), resulting in a 60-bp product in 30 μl of total reaction volume. A total of 5 μl was resolved by 3% agarose gel electrophoresis, with a 20-bp DNA standard (Qiagen) to ensure successful transformation. The remainder of PCR product was used for the pyrosequencing assay.

Pyrosequencing is a nonelectrophorectic method of DNA synthesis that uses a luciferase-based enzyme reaction to monitor DNA synthesis in real time and is widely used for genotyping (Fakhrai-Rad et aL, 2002). A pyrosequencing assay was adapted for use as an alternative to direct DNA sequencing for the identification of RNA edited isofonns. PCR product (25 μl) was mixed with 4 μl of streptavidin-coated Sepharose beads (Amersham Biosciences, Uppsala, Sweden) and 21 μl of binding buffer (10 mM Tris-acetate, 2 M NaCl, 1 mM EDTA, and 0.1% Tween 20, pH 7.6). The mixture was agitated at 1400 rpm for 10 min at room temperature and transferred to a MilliQ filter plate (Millipore, Molshelm, France). The plate was affixed to a vacuum, and liquid was removed. A 50-μl sample of 0,2 M NaOH was added to the plate, incubated for 1 min, and removed by vacuum. Next, 150 μl of 10 mM Tris-acetate, pH 7.6, was applied twice and removed immediately as before. A 54-l.d sample of annealing buffer (20 mM Tris-acetate and 2 mM Mg-acetate, pH 7.6) was mixed with 1.92 μl of 10 μM sense primer (5'-ATATCGCTGGATCGGTATGTAG-3') and used to resuspend the immobilized template in the filter plate. Thereafter, 45 μl of this mixture was transfered to a 96-well PSQ plate (Pyrosequencing, Uppsala, Sweden). The plate was covered, and the annealing reaction proceeded in a thermal cycler (MJ Research) at 80°C for 2 min. Enzyme and substrate reagents from the sodium nitroprusside reagent kit 5 x 96 (Pyrosequencing) were dissolved in 620 μl of high-purity water after reaching room temperature and loaded onto a PSQ 96 reagent cartridge (PyTosequencing). Of each dNTP, 200 μl was also loaded into compartments in the PSQ 96-reagent cartridge, as instructed by the manufacturer's protocol. The sample plate and cartridge were inserted into the $PSQ^{HA}96$ instrument, and analysis occurred automatically. The dispensation sequence entered into the $PSQ^{HA}96$ analysis program was GCAGCTAGTCGTAGAGTCTAGCT. The Biotage.com technical support team (http://www.biotage.comf)designsdispensationsequences using proprietary-software on request. PCR contamination was eliminated by fastidious PCR procedures, including the use of a filtered PCR cabinet, "DNA away" reagent (Molecular BioProducts, San Diego, CAl, filtered pipette tips and regular UV irradiation of all equipment. If present, contamination can be detected in negative controls or "blanks" and by the presence of extra peaks, indicating the presence of both G and A at a single site in a transcript. The presence of any apparent "heterozygosity" indicated contamination, thus ensuring the stringency of the method.

Animals and Drug Treatments. Male Sprague-Dawley rats (Harlan, Olac, UK) weighing 250 to 300 g received once-daily injec-

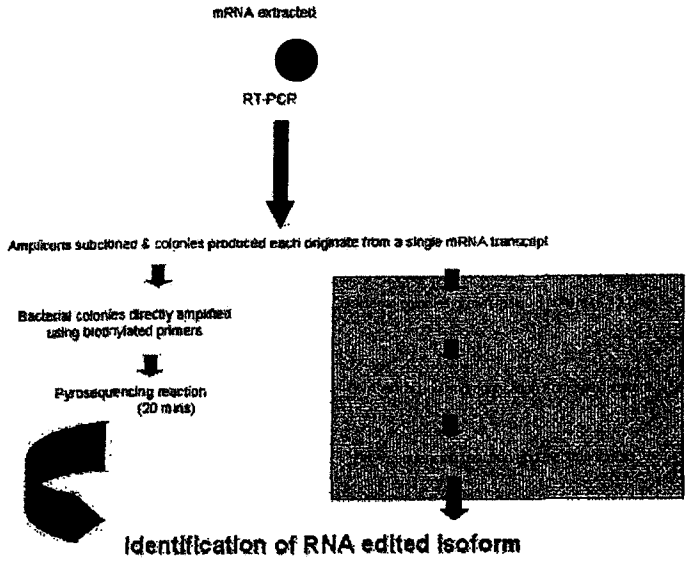

Fig. 2. A direct comparison between the new RNA-editing assay and the direct sequencing protocol. RNA is extracted by standard methods, and RT-PCR is performed to produce a mixed population of products, with varying degrees of RNA editing. The products can be analyzed by subcloning and then either sequencing or pyrosequencing. RT-peR products are subcloned, and colonies are chosen at random and tested for insert using PCR. Each colony represents Olle mRNA transcript within the heterogeneous population of mRNAs. For DNA sequencing (represented on the right side of the chart), positive colonies are incubated overnight in liquid culture, and DNA is extracted and purified before sequencing can be performed. The left side of the flowchart illustrates the steps involved in the new assay. The steps eliminated from the direct sequencing method are contained by the box on the right side of the flow chart. Therefore, the new assay eliminates the need for overnight bacterial culture, DNA extraction, purification, and sequencing, which considerably reduces time and expense, thereby facilitating the high-throughput processing of samples.

Hons intraperitoneally for 14 days with clozapine (25 mglkg), haloperidol (1 mgJkgl, risperidone (0.5 mglkg), chlorpromazine (15 rug! kg), or saline vehicle. Animals were killed 6 h after the final dose by a lethal injection of pentobarbital and perfused transcardially with phosphate-buffered saline. Brains were removed, frozen on dry *icel* alcohol slurry, and stored at −80°C. All procedures were carried out in accordance with United Kingdom ethical and legal regulations. Cryotome sections (IS μm) were cut from each block (corresponding coronal brain sections at the level of the hippocampus), and 12 sections were homogenized before RNA extraction.

Statistical Analysis: Statistical Power and Data Clustering. To date, inadequate attention has been given to appropriate statistical analysis of RNA-editing measures. In these experiments, the data are usually collected as multiple categorical measures (e.g., isoform type) per subject. Repeated measures from the same subject may not be independent. Greater similarity in the measures within a subject compared with measures in the rest of its group, is defined as data "clustering". Failure to recognize the presence of clustering in data can produce misleading standard errors, confidence intervals, and probability values. The degree of data clustering and cluster size (RNA molecules processed per animal) are critical, because clustering of observations within animals reduces statistical power. The presence and strength of clustering can be quantified by the intraclass correlation coefficient (ICC; the ratio of between-cluster variation to total variation).

If the presence of significant data clustering is confirmed by the ICC, additional observations must be added to the sample size needed if clustering was absent (ICC = 0). The effect of sample clustering on sample size calculations is quantified by the design effect (Snijders and Bosker, 1999l: design effect 1 + (n' − 1) X ICC, where ICC is the intraclass correlation and n' is the average cluster size. The following formula shows that the design effect increases with cluster size and ICC. Figure 3 illustrates the effect of different degrees of data clustering on statistical power achieved with different sample sizes (numbers of subjects and clones) considering large, medium, and small effect sizes. For example, if the ICC is 0.10 and there are 10 measures per animal, the design effect is 1 + (10 - 1) X 0.10 = 1.9. This means that to have the same power as an unclustered analysis (with ICC 0), almost twice the number of observations would be needed. If we needed "N" animals without clustering and then discovered that ICC > 0, the larger number of animals required would be the following: number of clusters = N x (Design Effect) N x ([1 + (n' - 1) x ICCD, where n' = average cluster size in observations per animal.

Therefore if we use five animals each with 10 clones for a total of 50 observations, and the ICC is 0.1, we would need 5 x (1 + 00 - 1) x 0.1) =5 X 1.9 =9.5 (Le., 9 or 10 animals). An ICC value of 0.1 may be considered medium clustering (Raudenbush and Liu, 2000), and with 10 observations per animal, a moderate amount of clustering almost doubles the necessary number of measurements to achieve the same statistical power.

Accurate estimation of statistical power must consider the possibility of clustered data. Traditional power analysis (Cohen, 1988, 1992) can estimate the required sample size in the absence of clustering, after which the design effect and corrected number of animals or patients can be calculated. If no pilot data are available to estimate an ICC, one may plot a range of cluster sizes and ICes, as shown in Fig. 3.

Statistical Analysis: Comparisons of Drug Treatments. Appropriate analyses of data that are significantly clustered include 1) deriving a summary statistic for each subject (e.g., level of editing at each site) and comparing the distribution of summary statistics; 2) using a robust estimate of variance that corrects for the effect of clustering on the standard error calculation; 3) using a random-effects model, that explicitly models the similarity of units within clusters; and 4) using a generalized estimating equation (GEE) that adjusts both standard errors and parameter estimates to allow for clustering (Kirkwood and Sterne, 2003a). GEE was used in the analyses of data generated in the current study.

We applied GEE models to clustered binary-dependent variables, a modeling framework that has not been used in previous studies of 5-HT_{2c} RNA editing. We modeled the effects of drug treatment on each dependent variable (either a predicted 5-HT_{2c} protein variant or a single site of RNA editing) using a GEE model with binomial family, logit link, and exchangeable correlation. In these models, saline was the reference category or odds ratio denominator Csaline odds ratio was considered to be 1.0). We described the data using binary variables, including presence or absence of specific 5-HT_{2C} protein isoforms and presence or absence of editing at specific sites A to E. The level of RNA editing at specific sites was calculated as the percentage edited fraction of the total number of molecules processed for that subject. To determine whether antipsychotic drug treatments alter 5-HT_{2C} RNA editing, we sampled a mean of 15 clones from each of 31 Sprague-Dawley rats (470 clones in total) and randomized to one of four antipsychotic treatments or a saline control treatment (see *Animals and Drug Treatrnentsl.* We tested for RNA editing differences between saline and drug treatment conditions by measuring protein isoform or RNA edit site frequencies. Protein isoform or RNA edit site categories with less than 5% frequency were not analyzed. The probability of detecting a false positive effect (α) was considered to be 0.05. Before using the GEE models, we tested for the presence of data clustering in the dependent variables using a likelihood ratio test.

Results

Until now, the most complete information of RNA-editing levels and frequencies of individual isoforms has been obtained by direct DNA sequencing of cloned RT-PCR products. Therefore, validation of the new high-throughput method has been performed using DNA sequencing. Substitution of DNA sequencing with the new assay reduces sample processing time from days to hours and reduces the cost of reagents to less than 10% of the usual price of DNA purification and sequencing.

Validation of the New Method. The key to the new method is the identification of RNA-edited transcripts by adapting a technique used for genotyping DNA, pyrosequencing. In our protocol, RT-peR of the 5-HT_{2c} RNA-edited region is required, and subsequently, the sampled amplicons are subcloned and single colonies (each of which corresponds to an individual transcript) are amplified directly to produce a template for identification using a pyrosequencing assay. The protocol is described in detail under *Materials and Methods* and is summarized in Fig. 2.

The new assay was validated using sequenced plasmid DNA Eleven sequenced clones were identified with 100% accuracy when both sense and reverse strands were analyzed by the new assay. Figure 4 displays the results of sequencing analyses and pyrosequencing identification of each clone, which were in complete agreement.

5-HT_{2c} RNA Editing in Rat Brain after Long-Term Antipsychotic Treatment. Application of the novel high-throughput 5-HT_{2C} RNA-editing assay in an original data set exhibits the usefulness of this assay and reveals that RNA editing is altered by antipsychotics. Haloperidol (1 mglkg) and risperidone (0.5 mg/kg) significantly altered 5-HT_{2c} RNA editing in rats treated with a 2-week regimen of daily injections.

Haloperidol decreased the level of editing at the D site [odds ratio (OR) = 0.37, p = 0.009,95% CI = 0.17 to 0.78],

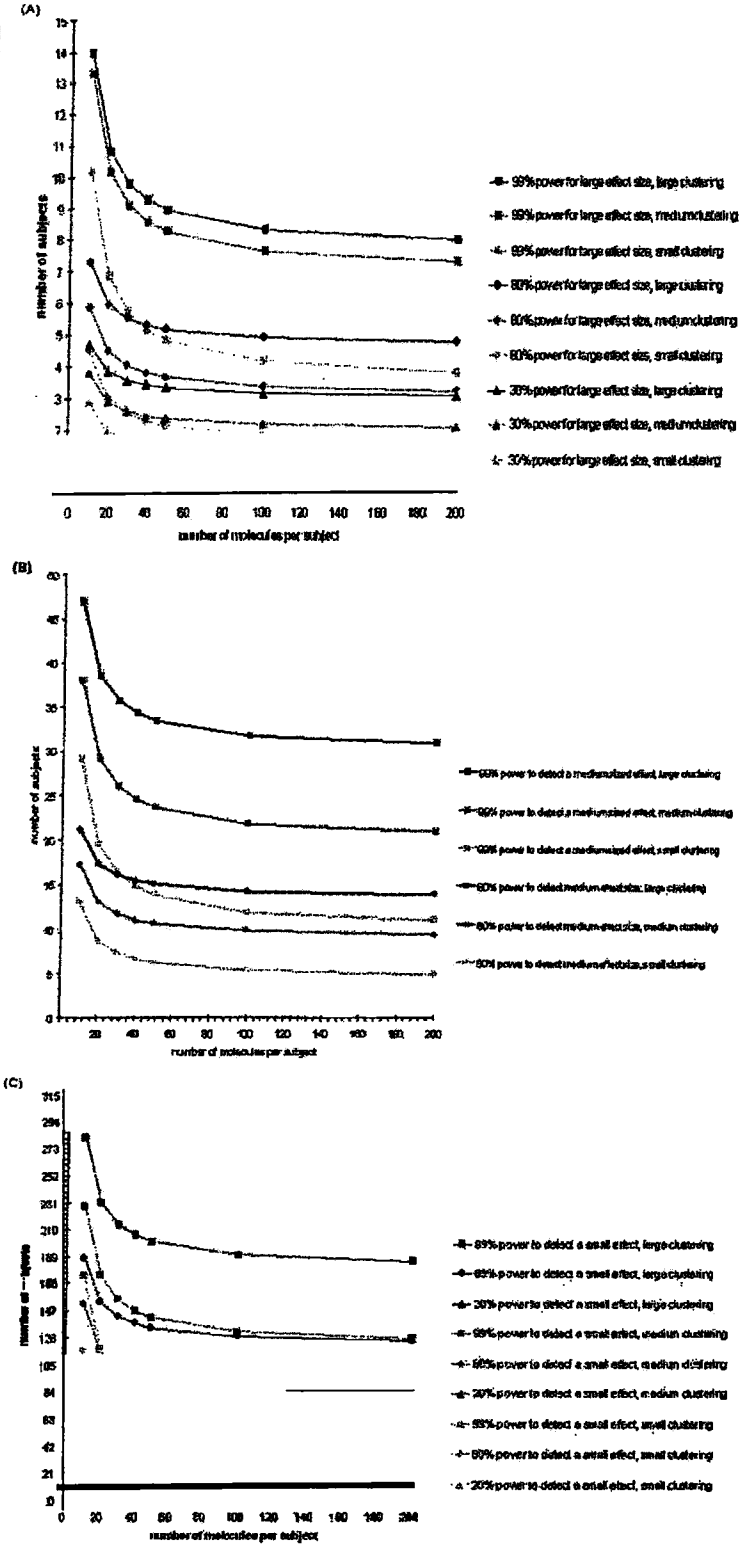

Fig. 3. Graphs to illustrate sample sizes required to detect small (A), medium (B), or large (C) effect sizes. The number of subjects and molecules processed per subject are compared at different levels of data clustering and statistical power. These graphs show that increased data clustering reduces statistical power, and therefore estimation of cluster size is especially critical if medium or small effect sizes are being measured.

leading to increased 5-HT_{2C} VNI isoform frequency (OR = *3.07,p* 0.017,95% CI = 1.22 to 7.71) and decreased 5-HT_{2C} VNV isoform frequency (OR = *OA8,p = 0.030,95%* CI = *0.25* to 0.93). By contrast, risperidone increased 5-HT_{2C} VNV isoform frequency (OR = *3.05, p = 0,005,* 95% CI = *lAO* to 6,65), Risperidone also tended to alter editing levels at the B, e, and D sites, although the direction ofthis effect relative to saline was different at each site, and these differences were less marked ($p < 0.10$), Neither clozapine nor chlorpromazine treatment led to statistically significant changes in any 5-HT_{2C} RNA-editing variable analyzed. Results are summarized graphically in Fig. 5.

RNA editing measurements with less than 5% frequency were excluded from the analysis (see *Materials Q.nd Methods).* Three 5-HT_{2C} protein isoforms had greater than 5% frequency: 5·HT,c VNl, *63/470:* 5.HT_{2C} VNV, *224/470:* and 5-HT_{2C} VSV, 112/470; therefore, these were included. When editing at individual sites was considered, only the unedited A and edited E sites were too infrequent to analyze. B-site editing levels were significantly clustered by animal after accounting for the treatment effect (ICC = 0.11, 95% CI = *0.03* to *0,32, X' = 7.01,p = 0.004).* Ofsix GEE models tested (Fig. 5), the full model probability was significant for the D site *(p = 0.0025)* and 5-HT_{2C} VNI *(p = 0.0192)* and 5.HT,c VNV *(p = 0.0001)* isoforms using Wald tests (4 df).

Analysis ofData Clustering. We demonstrate data analysis in the current study by considering RNA editing at the B site, which was measured as either edited or unedited. Effect size for a χ^2 test was considered to be Cohen's *w*. Our aim is for sufficient statistical power to detect small or medium effects, defined as $w = 0.1$ and 0.3, respectively (Cohen, 1992), and the probability ofdetecting a false-positive effect (α) was considered to be 0.05. We tested 31 animals (four treatment groups of six, and one group of seven animals), with 15 clones sampled per animal (31 X 15 = 465 clones). The ICC value was calculated to be 0.11. The effects ofsaline treatment on binary RNA-editing measures were compared separately with the effects ofeach drug treatment in a 2 x 2 χ^2 table (1 dD, with 7 versus 7 animals and 15 clones per animal, for a total of210 measures ofpresence or absence of an edited B site in each group. If we ignore clustering and assume that the measures are independent (ICC = 0), we estimate 99% power to detect a large and medium effect sizes but only 30% power to detect a small effect. However, the observations from each animal are not independent in this case, and we need to consider clustering when estimating statistical power. The design effect was calculated to be 1 + (n' 1) X *ICC* = I + (15 -]) X O.ll = 2.54. The number of clusters (or subjects) actually required for the statistical power calculated, when the ICC is really 0.11, is nln' [1 + (n' -]) x ICC] = *210/15* x 2,54 = 35.6 animals. The actual number of clones required for the sample power to be as predicted above is 35.6 x 15 = 533 (i.e., more than twice the number sampled). Dividing by the design effect suggests that the sample of210 clones has the statistical power ofa sample size of 83 independently sampled clones *(210/2,54* = 83), Standard power tables indicate that the genuine power to detect large effect sizes is 99%, for medium effects there is 77% power, and there is only 15% power to detect a small effect. Using Cohen's traditional criterion of80% as adequate power, we have adequate power only to detect large effects; if the effects are medium or small, the study is underpowered to detect changes in B-site editing.

Discussion

Development of the Novel Assay. We report a simple but powerful new method of facilitating the evaluation of RNA editing. Several genotyping strategies were attempted previously as alternatives to DNA sequencing to identify cloned transcripts (e.g., dot blotting and restriction fragment length polymorphism), but only our adapted pyrosequencing protocol produced accurate reproducible data with a straightforward procedure. Our pyrosequencing assay was validated in comparison with DNA sequence data and was found to be 100% accurate on both strands. The new method, which eliminates the need for overnight liquid culture and subsequent plasmid DNA extraction and purification stages, is therefore less labor-intensive and 90% less expensive than direct DNA sequencing. Hence, our new assay has replaced the use of primer extension and direct DNA sequencing in our research group.

Direct sequencing has been the preferred method in all the detailed studies of 5-HT_{2c}RNA editing to date because it is the only method currently available for determining 5-HT_{2c} isoform profiles (Sodhi et al., *2001;* Gurevich et al., *2002;* Dracheva et al., 2003; Yang et al., 2004). In addition, direct sequencing has been used for the identification of multiple RNA-editing sites produced by overexpression and subsequent hyperediting activity ofthe RNA editing enzyme APO-

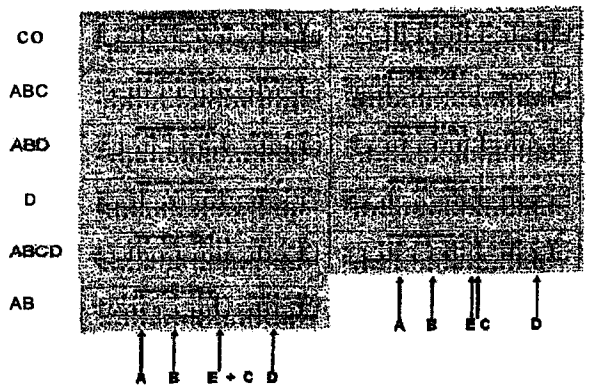

Fig. 4. Validation of the new high-throughput RNA-editing assay. Cloned plasmid DNAs from the 5-H'I'2CR RNA edited region were first sequenced using standard DNA sequencing techniques and subsequently processed using the new high-throughput assay. The pyrograms demonstrate the accuracy Of the new assay in the identification of each RNA-edited site. Each trace is labeled according to the sequencing data, which were replicated by the new method in every clone tested. The positions of sites vulnerable to RNA editing are indicated, and each peak. is labeled to provide the clone sequence.

BEe (Yamanaka et at, 1996) or for the identification of 16 naturally occurring edited sites in ADAR 2 (Dawson et al., 2004). The assay developed in this study can be adapted to quantify changes in these and other newly discovered RNA edited substrates. Although our protocol is optimized using pyrosequencing technology, similar DNA synthesis genotyp-iug platforms have been developed by GenoVoxx (Luebeck, Germany) and 454 Life Sciences (Branford, CT), which could be adapted for the assessment of RNA editing with minimal alterations to the current protocoL

Effects of Antipsychotic Drugs on 5-HT_W RNA Editing. This is the first report of altered 5-HT2c RNA editing after long-term treatment with antipsychotic drugs. Haloperidol, a commonly prescribed antipsychotic drug, reduces D-site editing, thereby increasing the frequency of the 5-HT_{2C} VNI isoform and reducing the frequency of the 5-HT_{2C} VNV isoform. Risperidone had the opposite effect. It is not clear why haloperidol, an antipsychotic drug with no affinity for 5-HT_{2C}R, would alter its RNA editing. Because chlorpromazine did not duplicate these effects, it seems that the common property of dopamine receptor antagonism is unlikely to be the mechanism. Although the decrease in RNA editing may be related to a generalized toxic effect of haloperidol, this is improbable because the reduction was not uniform across editing sites. Further studies including specific ligands for the receptors targeted by risperidone and haloperidol are required to determine which receptor(s) are involved in the mechanism producing the RNA editing changes observed. Clozapine did not cause any significant change in RNA editing; although our study had sufficient power (80%) to detect medium-sized effects (with the exception of changes at the B site, see *Analysis of Data Clustering* under *Materials and Methods),* it is possible that small effects were missed.

Altered D-site editing of the 5-HT_{2C}R is responsible for the

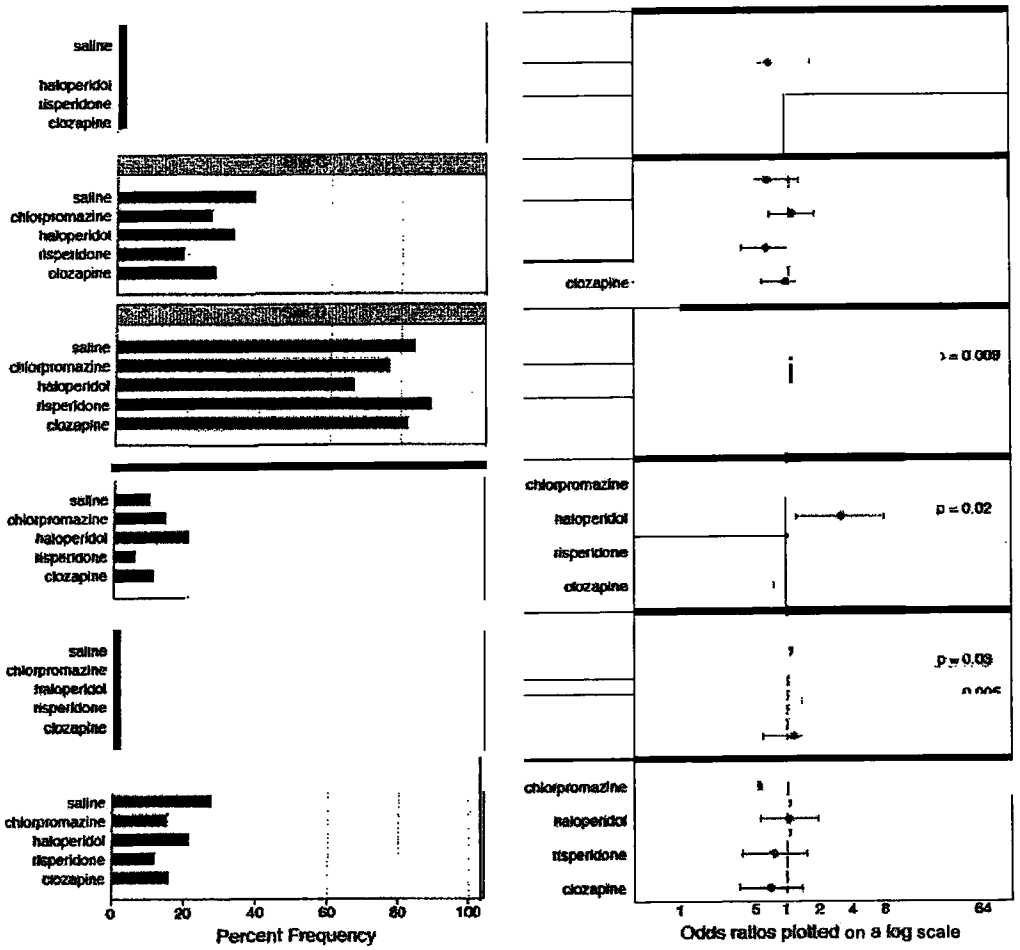

Fig. 5. The effects of antipsychotic drug treatments on 5-HT_{2c} RNA editing in rat brain. The bar chart on the left illustrates the percentage frequencies of each variable measured in the different treatment groups. Saline is the reference category for all models, B, C, and D are the percentage of editing levels. VNI, VNV, and VBV are the inferred 5-HT_{2c}-edited isoforms. The average measurements of each variable for the saline-treated group of animals can be compared directly with each drug-treatment group. The chart on the right illustrates the point OR values generated by GEE for each drug treatment group plotted on a logarithmic odds ratio scale (with the horizontal axis labeled on the OR scale). The 95% confidence intervals are indicated as bars around the point OR. The saline treatment is considered to have a point OR = 1. The statistical significance of the most robust changes is indicated.

change in 5-HT_{2C} VNI and 5-HT_{2C} VNV isoform frequencies. Because isoforms differing at the D site have similar G-protein coupling and constitutive activities in vitro (Herrick-Davis et al., 1999), it is reasonable to assume the editing changes resulting from haloperidol or risperidone treatment will be functionally silent, at least with respect to these components of 5-HT2CR activity. The D site of 5-HT_{2C}R is edited by ADAR 2 (Burns et al., 1997), and therefore the current data may indicate that haloperidol directly or indirectly alters the RNA editing machinery connected with ADAR 2 activity. ADAR 2 edits five members of the GluR gene family, In particular, the RNA edited QlR-site of the a-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor GluR2 subunit critically controls the calcium permeability of the associated ion channel (Lomeli et al., 1994). Manyadditional substrates for RNA editing are emerging (Levanon et al., 2004), and therefore a hitherto unidentified substrate could explain these findings.

Our data also have implications for neurogenetic studies of human disease. Postmortem findings have shown reduced GluR2 Q/R site editing and reduced 5-HT_{2C} RNA editing (at all sites) in schizophrenia (Akbarian ot al., 1995; Sodhi et al., 2001). Although many of the patients in the latter study were not treated with haloperidol (Sodhi et al., 2001), it is possible that long-term antipsychotic treatment could have contributed to the RNA editing reduction in subjects with schizophrenia compared with the control subjects. However, the reduced RNA editing in subjects with schizophrenia was observed at all editing sites, and not just at the D site as in the haloperidol-treated rats. Additional studies of animals administered drug doses with greater equivalence and clinical relevance will clarify these findings, but the current data indicate that caution is required in the interpretation ofRNA editing changes in tissue taken from patients exposed to long-term antipsychotic drug treatments. Furthermore we demonstrate that in vivo RNA editing can be modulated by an environmontal, nongenetic factor (i.e., drugs) to interrupt the linear relationship between genotype and phenotype, This would not be detected by genomic DNA analysis and therefore could produce contradictory molecular genetic data in studies of complex hereditary diseases, such as schizophrenia.

Statistical Power and Data Clustering. Statistical power estimates of the current study deal with data clustering and support the need for a high-throughput method to measure RNA editing. It is estimated that to achieve 80% power to detect a small effect with medium clustering, the minimum number ofsubjects required would be 84 when 200 RNA molecules from each subject are tested. Although this is a large sample, using the new methodology, 16,000 clones could be processed using 178 x 96-well plates. The same task using DNA sequencing would be prohibitively lengthy and expensive. Circumventing this problem by pooling extracted RNA or cDNA from animals in a group to compare differences between groups ignores the environmental differences between animals that could create clustering in some variables measured. Clustering was observed in the current data when the level ofB site editing was measured. Pooling ofmRNA or cDNA would therefore preclude estimations of clustering for reliable statistical analyses.

Previous analyses ofRNA editing data have proceeded as if the transcripts were statistically independent of the subject from which they were sampled (Sodhi et al., 2001). This is equivalent to assuming that the ICC = 0. Caution is warranted with this approach, because 1) the ICC confidence interval in previously published and current data often have an upper limit invalidating the assumption ofindependence, and 2) it can be argued that the appropriate null hypothesis for using a test that assumes independence should be that the ICC is less than a specific negligible value, which is analogous to a minimum-effect null hypothesis (Murphy and Myors, 2003). These statistical calculations depend on an accurate estimation of the ICC or clustering (Zou and Donner, 2004),

The presence ofclustering will reduce the statistical power ofa given sample size compared with the same sample size in which clustering is absent (i.e., the measures are independent). This reduction will depend on the level of the clustering present. With medium-sized data clustering (lCC = 0, U, the study had 99% power to detect large effects (effect size, w = 0.5) and greater than 95% power to detect medium effects (w = 0.3). However to detect small effects (w = 0,1), the presence of medium-sized clustering reduces the statistical power to approximately 25%, These statistical power estimations were made from the graphs in Fig. 3 and published tables of statistical power (Cohen, 1988). Figure 3 illustrates that if the ICC is large (>0.3), power rapidly becomes insensitive to additional clones per animal and that increased power is best achieved by the addition of animals or subjects (Hsieh, 1988). The use of models that allow for clustering should have at least 30 clusters (Kirkwood and Sterne, 2003b). In smaller samples, simpler analyses of summary statistics should be considered.

In conclusion, we have adapted a simple genotyping method to identify RNA edit variants and have addressed statistical issues associated with this research problem. There seems little doubt that RNA editing is widespread and is an important source of protein diversity (Levanon et al., 2004). The RNA-editing events detected in mammals have profound effects on protein function, and these are yet to be fully explored in the etiology of neurodegenerative and psychiatric disorders (Akbarian et al., 1995; Niswender et al., 2001; Sodhi et al., 2001; Gurevich et al., 2002; Dracheva et al., 2003). Furthermore, the current study provides evidence that risperidone and haloperidol alter 5-HT2C RNA editing in different ways that may be related to their efficacy as antipsychotics. Therefore, we have developed an accurate, high-throughput tool for greater exploration of the pathological consequences ofaltered RNA editing. The importance ofRNA editing is likely to increase as new substrates are identified, and it is possible that the novel RNA editing assay could be adapted in the future for the purpose of diagnosis or drug development.

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References

Akbarian S, Smith MA, and Jones EG (1995) Editing for an AMPA receptor subunit RNA in prefrontal cortex and striatum in Alzheimer's disease, Huntington's disease and schizophrenia. *Brain Res 699:297-301.*

Bmus eM, Chu H, Rueter SM, Hutchinson LK, Cantoll H, Sanders-Bush E, and

Emeson RB (1997) Regulation ofserotonin-2C receptor G-protein coupling by RNA editing. *Nature (Lond) 387:303-308.*

Cohen J (1988) *Statistical Power Analysis for the Behavioral Sciences.* Erlbaum Associates, Hillsdale, NJ.

Cohen J (1992) A power primer. *Psychol Bull* 112:155-159.

Dawson TR, Sansam CL, and Emeson RB (2004) Structure and sequence determinants required for the RNA editing ofADAR2 substrates. *J Biol Chem* 279:4941-4951.

Dracheva S, Elhakem SL, Marcus SM, Siever LJ, McGurk SR, and Haroutunian V (2003) RNA editing and alternative splicing of human serotonin 2C receptor in schizophrenia. *J Neurochem 87:1402-1412.*

Fakhrai-Rad H, Pourmand N, and Ronaghi M (2002) Pyrosequencing: an accurate detection platform for single nucleotide po[ymorphisms. *Hum Mutat* 19:479-485.

Gott JM (2003) Expanding genome capacity via RNA editing. C *R Biol* 326:901-908.

Gurevich I, Tamir H, Arango V, Dwork AJ, MannJJ, and Schmauss C (2002) Altered editing of serotonin 2C receptor pre-mRNA in the prefrontal cortex of depressed suicide victims. *Neuron* 34:349-356.

Herrick-Davis K, Grinde E, and Niswender eM (J999) Serotonin 5-HT2C receptor RNA editing alters receptor basal activity: implications for serotonergic signal transduction. *J Neurochem 73:1711-1717.*

Hsieh FY (1988) Sample size formulas for intervention studies with the cluster as the unit ofrandomizalion. *Stat Med 8:1195-1201.*

Kawahara Y, Ito K, Sun H, Aizawa H, Kanazawa I, and Kwak S (2004) Glutamate receptors: RNA editing and death of motor neurons. *Nature (Lond) 427:801.*

KirkwilOd BR and Sterne JAC (20030.) Analysis of clustered data, in *Essentials of Mf.dical Statistics,* pp 355-370. Blackwell Science, Oxford.

Kirkwood BR and Sterne JAC (2003b) *Essentials ofMedical Statistics,* pp 423-424. Blackwell Science, Oxford.

Levanon EY, Eisenberg E, Ye[in R, Nemzer S, HaBegger M, Shemesh R, Fligelman ZY, Shoshan A, Pollock SR, Sztybel D, et al. (2004) Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat Biolechnol* 22: 1001-1005.

Lomeli H, Mosbacher J, Melcher T, Hoger T, Geiger JR, Kuner T, Monyer H, Higuchi M, Bach A, and Seeburg PH (1994) Control ofkinetic properties ofAMPA receptor channels by nuclear RNA editing. *Science (Wash DC) 266:1709-1713.*

Maas S, Patt S, Schrey M, and Rich A (2001) Underediting of glutamale receptor GluR-B mRNA in malignant gliomas. *Proc Natl Acad Sci US.498:14687-14692.*

Murphy KR and Myors B (2003) *Statistical Power Analysis: A Simple and General Model (I). Traditional and Modern Hypothesis Tests.* Lawrence Erlbaum, Mahwah, NJ.

Niswender CM, Copeland SC, Herrick-Davis K, Emeson RB, and Sanders-Bush E (1999) RNA editing of the bwnan serotonin 5-hydroxytryptamine 2C receptor silences constitutive activity. *J Biol Chem* 274:9472-9478.

Niswender eM, Herrick-Davis K, Dilley GE, Meltzer HY, Overholser JC, Stockmeier CA, EmesoD RB, and Sanders-Bush E (2001) RNA editing of the human serotonin 5-HT2C receptor. alterations in suicide and implications for serotonergic pharmacotherapy, *Neuropsychopharmacology* 24:478-491.

Raudenbush SW and Liu X (2000) Statistical power and optimal design for multisite randomized trials. *Psychol Methods* 5:199-213.

Snijders T and Bosker R (1999) *Multilevel Analysis: An Introduction to Basic and Advanced Multilevel Modeling.* Sage Publications, Thousand Oaks. CA.

Sodhi MS, Burnet PW, Makoff AJ, Kerwin RW, and Harrison PJ (2001 RNA editing of the 5-HT_{2C} receptor is reduced in schizophrenia. *Mol Psychiatry* 6:373-379.

Yamanaka S, Poksay KS, Driscoll DM, and Innerarity TL (1996) Hyperediling of multiple cytidines of apolipoprotein B mRNA by APOBEC-1 requires auxiliary protein(s) but not a mooring sequence motif. *J Biol Chem 271:11506-11510.*

YangJH, Luo X, Nie Y, Su Y, Zhao Q, Kabir K, Zhang D, and Rabinovici R (2003) Widespread inosine-containing mRNA in lymphocytes regulated by ADARI in response to inflammation. *Immunology 109:15-23.*

Yang W, Wang Q, Kanes SJ, Murray .IM, and Nishikura K (2004) Altered RNA editing of serotonin 5-HT2C receptor induced by interferon: implications for depression associated with cytokine therapy. *Brain Res Mol Brain Res* 124:70-78.

Zou G and Donner A (2001) Confidence interval estimation of the intraclass correlation coefficient for binary outcome data. *Biometrics* 60:807-811.

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Research Article

Identification and relative quantification of adenosine to inosine editing in serotonin 2c receptor mRNA by CE

A new method has been developed allowing the identification and relative quantification of different forms of mRNA after RNA editing. This method was applied to the serotonin 2c receptor mRNA that potentially exhibits 32 different forms after adenosine to inosine editing at five different sites located in a row of 13 nucleotides. CE was used to characterize fluorescently labeled ssDNA molecules on the basis of their conformational polymorphism. The relative amount of these 32 mRNA forms has been estimated by measuring the fluorescence intensity of each individual DNA strand. Accuracy of quantification was established by diluting one form into another or into a mixture of cDNA, showing linear and precise proportion of each form ($0.06 < SD < 0.39\%$). In mouse brain tissue samples, up to 23 different mRNA forms were characterized and quantified, even for forms representing less than 1% of the mixture.

Keywords:
5-HT2cR mRNA / CE / RNA editing / Serotonin / SSCP DOI 10.1002/elps.200600698

1 Introduction

Editing of ribonucleic acid (RNA) is an important mechanism in post-transcriptional regulation of gene expression that allows the cell to produce multiple RNA molecules from a unique RNA transcript [1]. Among the different kinds of RNA editing, specific adenosine (A) to inosine (I) conversion in messenger RNA (mRNA) is carried out by adenosine deaminases that act on RNA (ADAR) [1]. Three ADARs have been described in vertebrates; two of which (ADAR 1 and ADAR 2) catalyze the hydrolytic deamination of peculiar A to I in well-defined double-stranded RNA structures, converting adenine to hypoxanthine [1]. During mRNA translation, I is supposedly read as a guanosine (G), thus leading to potential amino acid changes in the encoded protein. The best-studied substrate for ADAR 1 and ADAR 2 is probably the pre-mRNA of the receptor subtype 2c of serotonin (5-hydroxy tryptamine receptor 2c, 5-HT2cR) [1, 2]. In this pre-mRNA, as many as five A can be edited by these ADARs, leading to 32 different 5-HT2cR mRNA, and 24 different 5-HT2cR, with amino acid substitutions in the second intracellular loop of this G-protein-coupled receptor (GPCR) (Fig. 1). Therefore, due to its localization, this change in amino acids could significantly alter the 5-HT2cR coupling to G-proteins, as well as desensitization and trafficking of the receptor [3–8]. Briefly, an unedited receptor (I^{156}-N^{158}-I^{160}, see Fig. 1a) activates the G-protein coupling more efficiently than a fully edited one (V^{156}-G^{158}-V^{160}, see Fig. 1a) upon agonist stimulation [9, 10]. The 5-HT2cR is widely distributed in the central nervous system and is thought to play a major role in appetite and sexual behavior, as well as in mood regulation [2]. The level of editing appears to be brain structure-dependent and several alterations of this editing have been described in some mental disorders in humans, as well as upon drug administration in mice [2, 11–15]. Sites of editing can also be removed together with the alternatively spliced exon Vb within the 5-HT2cR mRNA (Fig. 1b) [15]. Indeed, editing influences alternative splicing by disrupting in exon Vb, a splicing silencer that interacts with a small nucleolar RNA (snoRNA), the expression of which is impaired in the Prader–Willi syndrome, leading to a defect in 5-HT2cR pre-mRNA processing that contributes to this syn-

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Abbreviations: A, adenosine; **ADAR,** adenosine deaminase that act on RNA; **cDNA,** complementary deoxyribonucleic acid; **CP,** choroïd plexus; **GPCR,** G-protein-coupled receptor; **DNA,** deoxyribonucleic acid; **FC,** frontal cortex; **G,** guanosine; **5-HT2cR,** 5-hydroxy tryptamine receptor 2c; **I,** inosine; **mRNA,** messenger RNA; **RFU,** relative fluorescence unit; **RNA,** ribonucleic acid; **RT,** reverse transcription; **snoRNA,** small nucleolar RNA

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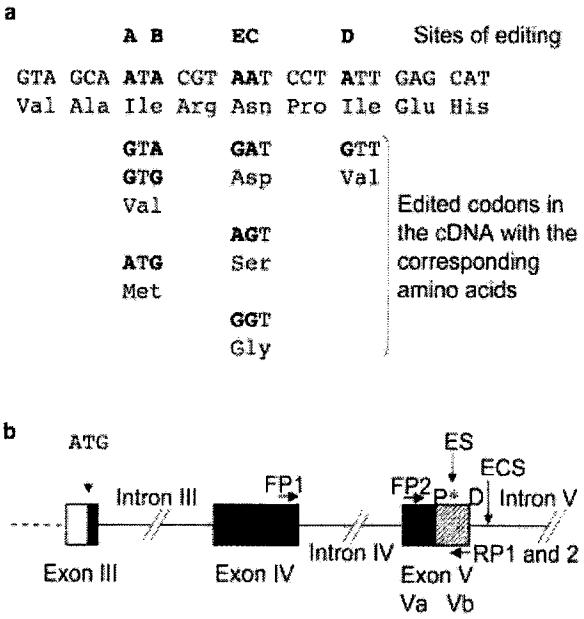

Figure 1. (a) Nucleotide and amino acid changes in 5-HT2cR sequences. Genomic DNA sequence (or nonedited cDNA) is aligned with the amino acid sequence located within the 5-HT2cR second intracellular loop. Positions of the five sites of editing (A, B, C, D and E) are indicated above this sequence in which bold A stands for nonedited and bold G stands for A to I replacement. This RNA editing leads to 32 cDNA different forms and 24 protein variants. Amino acids which are potentially modified are in positions 156, 158 and 160 of the full length receptor sequence. (b) Partial structure of the mouse 5-HT2cR gene. ES stands for editing site, the sequence of which is written in (a); ECS stands for editing site complementary sequence located in the 5′ region of intron V; FP1 and RP1 specify the positions of respectively forward and reverse primers used for the first PCR leading to a 250 bp product; FP2 and RP2 specify the positions respectively of forward and reverse primers used for the second PCR nested in the first one, leading to the final 153 bp product, FP2 and RP2 being labeled in 5′ with FAM and VIC fluorophores respectively; P and D in exon V specify the location of proximal and distal donor splice sites respectively, subdividing exon V in exons Va (black box) and Vb (hatched box) (see Fig. 1 in ref. [15] for more details). Note that the shortest 5-HT2cR mRNA arising from the use of the proximal donor site cannot be amplified after RT-PCR, due to the absence of complementary sequences to RP1 and RP2.

drome ([15] and see also Fig. 1b). Therefore, for all these reasons, a fast and accurate method of identification and relative quantification of the 32 different 5-HT2cR mRNA, would be of primary importance for the study of any mechanism related to the function of this serotonin GPCR. So far, two methods have been used for this purpose, but they have significant shortcomings. The first method, based upon primer extension in the presence of an adequate dideoxy-nucleotide, allows estimation of the percentage of editing of one given site, but does not give any information on the relative proportion of each mRNA form [3, 16]. The second involves sequencing of a large number of complementary DNA (cDNA) clones of the 5-HT2cR mRNA mixture; a number far too small to give statistically significant results, whatever the method used for DNA sequencing [14, 16]. In addition, both methods are time-consuming and expensive. Here, we describe a new method for the identification and relative quantification of A to I editing in mRNA. This method is based upon direct analysis of cDNA by CE, in conditions established for discriminating the edited forms that correspond to the different 5-HT2cR mRNA. This analysis takes advantage of the unique electrophoresis mobility of each strand of the different cDNA, due to single-strand conformational polymorphism (SSCP) of the DNA [17–19].

2 Materials and methods

2.1 RNA isolation, cDNA synthesis and RT-PCR

Immediately after removal, rat or mouse brains (Wistar and Balb/C both from Charles River Laboratories France, Domaine des oncins BP 0109, 69592 LÁrbresle CEDEX) were frozen in isopentane at −30°C. They were then sliced up in 300 μm thick coronal sections. Frontal cortex (FC) and choroïd plexus (CP) were removed from these sections with a 1.5 mm diameter punch, and conserved into a solution of "RNA Later" (Ambion Diagnostics 2130 Woodward Austin, TX 78744-1832, USA) until RNA extraction. Total RNA was extracted, then treated with DNAse I using the "Nucleospin® RNA II" kit (Macherey-Nagel BP 135, Hoerdt, France). RNA quality was controlled with a Bioanalyzer 2100 (Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA, USA) and its amount was quantified by measuring the absorbance at 260 nm using a Nanodrop™ spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription (RT) of 5-HT2cR mRNA was carried out with 15 units of reverse transcriptase (Thermoscript Invitrogen™ from Invitrogen SARL, Cergy Pontoise CEDEX, France) starting from 0.5 μg of total RNA, using either 10 nmol of a specific reverse primer (5′-TCGTCCCTCAGTCCAATCAC-3′) that hybridizes to exon VI in a sequence located near its 5′-end, or 50 nmol of oligo dT as primer, following the manufacturer's protocol. A 250 bp cDNA fragment was then amplified by PCR with 0.5 unit of Platinum® *Taq* DNA polymerase (Invitrogen™) in a final volume of 25 μL, using a forward primer 1 (5′-TGTCCCTAGCCATTGCTGATATGCT-3′) and a reverse primer 1 (5′-GCAATCTTCATGATGGCCTTAGTCCG-3′), both at 0.2 μM final concentration that hybridize to exon IV, and exon V respectively, in sequences located near their 3′-end (Fig. 1b). These primers were used for both rat and mouse, the 250 bp fragment being identical in both species. After an initial denaturation step at 94°C for 2 min, the PCR was brought to its final point after 35 cycles (30 s at 94°C; 30 s at 55°C; 30 s at 72°C), and a final elongation step of 5 min at 72°C.

2.2 Preparation of the 32 cDNA forms

Fragments of 5-HT2cR cDNA, 250 bp long, were obtained by RT-PCR starting from RNA extracted from rat CP and total brain, as described above. They were then cloned into the TA-cloning vector pCR®4-TOPO® with the TOPO TA Cloning® Kit (Invitrogen™). After transformation of *Escherichia coli* (TOP10 strain), 103 clones were picked and amplified. Plasmids were then prepared by standard procedures and purified with the "Nucleospin® Plasmid" kit (Macherey-Nagel). The amount of each of them was quantified by measuring the absorbance at 260 nm using a Nanodrop® spectrophotometer. For each plasmid, the 250 bp cDNA fragment was sequenced by the dideoxy sequencing method with a MegaBACE 1000 (GE Healthcare UK, Amersham Place, Little Chalfont Buckingamshire, HP 7 9NA, England) following the manufacturer protocol. By this strategy, 13 different 250 bp-long cDNA were obtained, representing 13 out of the 32 possible forms of 5-HT2cR mRNA, namely the NE, A, D, AB, AC, AD, CD, DE, ABD, ACD, ABCD, ABDE and ACDE forms. The 19 lacking cDNA were obtained by site-directed mutagenesis using the "GeneTailor™ Site-Directed Mutagenesis System" (Invitrogen™).

2.3 Separation of single-strand cDNA fragments by CE

For analysis of the 32 different cDNA fragments by mean of their SSCP, DNA was reamplified by PCR either from the 32 different cDNA inserted into plasmids, or from the mixture of 5-HT2cR cDNA fragments, 250 bp long, obtained by RT-PCR. This second PCR was carried out with HPLC-purified fluorescent primers with 0.5 unit of Platinum® *Pfx* DNA polymerase (Invitrogen™) in a final volume of 25 μL, and led to new 153 bp-long fragments, labeled on both 5′-ends with FAM (forward strand) and VIC (reverse strand) fluorescent dyes (forward primer 2: FAM-TTTGTGCCCCGTCTGGAT-3′, from Invitrogen™, and reverse primer 2: VIC-GCAATCTTCATGATGGCCTTA-3′, from Applied Biosystems, Warrington, Cheshire, UK), both at 0.3 μM final concentration. The position of the primers is indicated in Fig. 1b. Here again, after an initial denaturation step at 94°C for 2 min, the PCR was brought to its final point after 35 cycles (15 s at 94°C; 30 s at 55°C; 20 s at 68°C), and a final elongation step of 5 min at 68°C. In these conditions, the PCR limiting factor was the availability of both fluorescently labeled primers, as shown by CE (see below in Section 3.1).

Fluorescent PCR products (1 μL after a dilution of 1/30 to 1/200) and deionized formamide (11 μL) were added to a mixture of 13 different migration standards (0.5 μL) used for calibrating the electrophoresis in capillaries [20, 21]. These migration standards were specifically built for this purpose, and were labeled with ROX™ fluorescent dye (from Invitrogen™) as follows: 138 bp fragments of mouse forms ABCDE, DE and ACD were amplified by PCR with ROX™-labeled forward primer ROX-TTTGTGCCCCGTCTGGAT-3′ and reverse unlabeled primer 5′-GCCTTAGTCCGCGAATTG-3′; 153 bp fragments of mouse forms ABCDE, ABC, ACE and NE were amplified by PCR with unlabeled forward primer 5′-TTTGTGCCCCGTCTGGAT-3′ and ROX™-labeled reverse primer ROX-GCAATCTTCATGATGGCCTTA-3′; 153 bp fragments of mouse forms ABCDE, ACD, DE, A and ABCE as well as a 153 bp fragment of human form ABE were amplified by PCR with ROX-™labeled forward primer ROX-TTTGTGCCCCGTCTGGAT-3′, and unlabeled reverse primer 5′-GCAATCTTCATGATGGCCTTA-3′. Samples were denatured for 2 min at 90°C then immediately chilled on ice. Nondenaturing electrophoresis was carried out in an ABI PRISM® 3100-*Avant* Genetic Analyzer (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA) through 80 cm-long capillaries filled with 7% "POP™ Conformational Analysis Polymer" (Applied Biosystems), 1X Tris-borate-EDTA and without glycerol. After a prerun carried out at 15 kV for 3 min, samples were injected for 15 s at 2 kV, and electrophoresis was performed for 105 min at 15 kV, at a strictly controlled temperature of 24°C. Under these conditions, each of the 64 different ssDNA (32 FAM-labeled and 32 VIC-labeled) was resolved as almost a single peak and the individual retention times were used for identification.

2.4 Identification and relative quantification of each cDNA form in a complex mixture

Raw data given by the ABI PRISM® 3100-*Avant* Genetic Analyzer were extracted for signal processing by the PeakFit® (v4.11) software (Ritme Informatique, Paris, France). For each electrophoresis profile given by FAM-, VIC- or ROX-labeled fragments, this processing smoothes the signal and corrects the baseline, for determination of the migration times corresponding to the maximum intensity of fluorescence. This allowed precise positioning of the reference cDNA relative to the electrophoresis profile given by the complex mixture of different cDNA contained in a given sample. Next, the values given by the sample and by each individual cDNA form were normalized to have the same profile area defined as 100%. Starting from the assumption that the editing profile corresponds to the sum of the 32 forms potentially present in the sample, it is necessary to determine the coefficients to be applied to each of them to give, as a function of time (from 0 to n scans, with 6.22 scans/s):

$$F(\text{Sample}) = a \times F(\text{form A})_{t=0} + b \times F(\text{form B})_{t=0} + \cdots + af \times F(\text{form ABCDE})_{t=0}$$

$$F(\text{Sample}) = a \times F(\text{form A})_{t=1} + b \times \text{F(form B)}_{t=1} + \cdots + af \times F(\text{form ABCDE})_{t=1} + \cdots$$

$$F(\text{Sample}) = a \times F(\text{form A})_{t=n} + b \times F(\text{form B})_{t=n} + \cdots + af \times F(\text{form ABCDE})_{t=n}$$

where F is the sum of VIC plus FAM normalized relative fluorescence unit (RFU), a is the coefficient to be applied to form A, b is to form B and so on, up to af for form ABCDE.

The resolution of these equations with 32 unknown factors is possible as a function of time using a matrix calculus in Excel (Microsoft Corporation, Redmond, WA, USA). A value expressed as a percent of the total area is thus obtained for each form present in a given sample containing up to 32 different forms. It is then possible to estimate the error due to this calculation, by means of a correlation coefficient between the electrophoresis profile given by the sample and the reconstituted profile given by the numerical results.

3 Results

3.1 Separation of single-strand cDNA fragments by CE

Thirty-two cDNA fragments corresponding to the potentially existing forms of 5-HT2cR mRNA were first cloned in plasmids in order to be used as references. These cDNA were designated according to the position of the five potentially edited sites (Fig. 1a). For example, "A" specifies the form edited on site A only. Similarly "B" specifies editing of site B only. "AB" specifies the form edited on the sites A and B simultaneously, and so on up to "ABCDE" that specifies the fully edited form. NE indicates the non-edited form. From these plasmids, 32 fragments of 153 bp were amplified by PCR with forward and reverse primers labeled at the 5′-end with FAM and VIC fluorescent dyes, respectively. The two ssDNA of each cDNA form were then separated by CE, in nondenaturing conditions on the basis of their conformational polymorphism. Under the conditions used, 30 out of the 32 possible forms gave unique electrophoresis profiles with unique migration times for at least one of the two ssDNA (Fig. 2 and Table 1). In the conditions used, only the NE and E forms exhibited identical electrophoresis properties, with identical migration times for both strands, expressed in number of scans with 6.22 scans/s (Table 1). For each of the different forms, the reverse strand consistently exhibited greater electrophoresis mobility than the corresponding forward strand (Fig. 2). In addition, all reverse strands migrated ahead of all forward strands, and consequently the reverse strands gave a superior separation (Table 1). The fastest reverse strand was the ABCDE form, detected 433 scans after the first ROX™-labeled migration standard. The NE and E forms were the slowest reverse strands, detected 2479 scans later (2912 minus 433 in Table 1). The fastest forward strand was also the ABCDE form, detected 2985 scans after the first ROX™-labeled migration standard, while the slowest was the ABE form, detected 1288 scans later (4273 minus 2985 in Table 1). The fluorescence intensity of the FAM-labeled strand was always higher than that of the VIC-labeled strand, as expressed in RFU (Fig. 2). This difference in intensity is mainly due to the detection system used by the ABI PRISM 3100-*Avant* Genetic Analyzer that stimulates fluorescence with a single laser beam. This laser primary emission lines are at 488 and 514.5 nm that do not correspond exactly to the FAM and VIC maximum excitation wavelengths (492 and 538 nm respectively). Moreover, the emitted fluorescence is analyzed in a way designed to eliminate the overlapping of FAM and VIC fluorescence recorded at their maximum emission wavelengths (517 and 554 nm respectively). However, the ratio between their RFU was identical for each of the 32 forms. The area of a given form was determined as the sum of the RFU recorded for each scan, and normalized to 100% (see Section 2.4), whatever the shape and the number of peaks exhibited by this form (Fig. 2). This normalization to 100% allows overcoming the difference in fluorescence intensity recorded for FAM and VIC fluorophores. Lastly, in the conditions of PCR used,

Table 1. Migration times of the VIC-labeled reverse and FAM-labeled forward strands

Forms	VIC-labeled reverse strands		FAM-labeled forward strands	
	Scan numbers	CV (%)	Scan numbers	CV (%)
NE	2912	0.33	3278	0.02
A	2109	0.23	3804	0.02
B	2494	0.12	3365	0.05
C	2704	0.22	3193	0.02
D	1931	1.42	3417	0.02
E	2912	0.18	3279	0.02
AB	2423	0.45	3912	0.02
AC	2161	0.36	3516	0.06
AD	2460	0.06	3742	0.05
AE	2584	0.27	3967	0.03
BC	2116	0.37	3212	0.03
BD	2424	0.22	3542	0.04
BE	2490	0.05	3864	0.05
CD	2037	0.64	3218	0.02
CE	2846	0.37	3349	0.02
DE	1939	0.74	3398	0.02
ABC	1123	0.41	3797	0.05
ABD	1330	0.44	3820	0.07
ABE	1988	0.78	4273	0.01
ACD	2846	0.20	3121	0.02
ACE	2415	0.36	3992	0.03
ADE	2218	0.25	3816	0.15
BCD	1444	0.42	3247	0.02
BCE	2241	0.32	3397	0.02
BDE	1577	0.24	3283	0.17
CDE	2449	0.04	3604	0.02
ABCD	445	1.51	3179	0.01
ABCE	972	0.47	4184	0.02
ABDE	2690	0.08	3452	0.14
ACDE	663	0.46	3282	0.28
BCDE	938	0.85	3054	0.12
ABCDE	433	1.24	2985	0.04

They are expressed in number of scans (at 6.22 scans/s), between the fastest ROX™-labeled migration standard and each of the 32 individual forms listed in the first column. CV indicates the coefficient of variation for each value, calculated as SD/mean ($n = 6$).

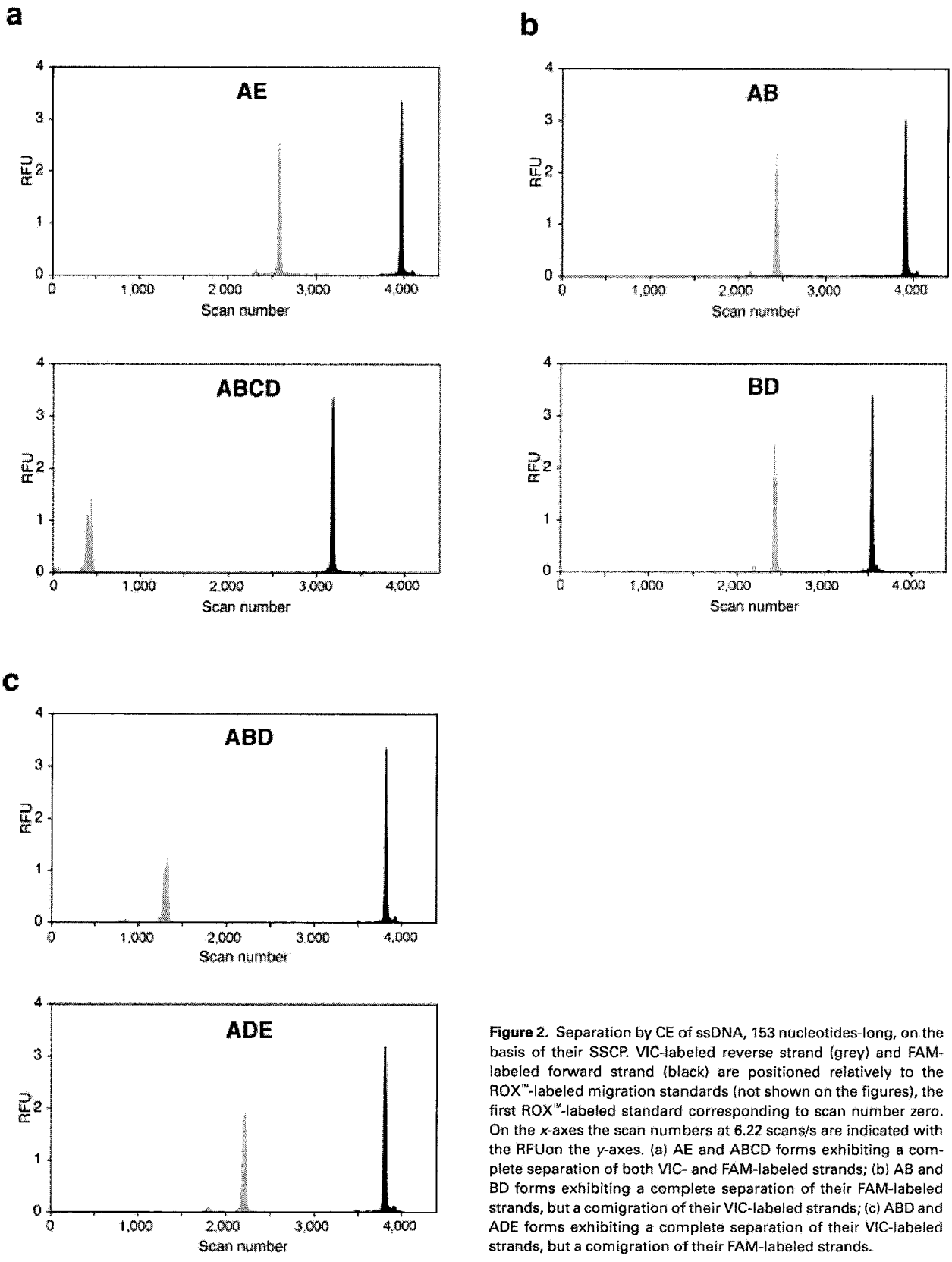

Figure 2. Separation by CE of ssDNA, 153 nucleotides-long, on the basis of their SSCP. VIC-labeled reverse strand (grey) and FAM-labeled forward strand (black) are positioned relatively to the ROX™-labeled migration standards (not shown on the figures), the first ROX™-labeled standard corresponding to scan number zero. On the *x*-axes the scan numbers at 6.22 scans/s are indicated with the RFUon the *y*-axes. (a) AE and ABCD forms exhibiting a complete separation of both VIC- and FAM-labeled strands; (b) AB and BD forms exhibiting a complete separation of their FAM-labeled strands, but a comigration of their VIC-labeled strands; (c) ABD and ADE forms exhibiting a complete separation of their VIC-labeled strands, but a comigration of their FAM-labeled strands.

neither labeled primers were detected by CE, indicating that both of them were completely incorporated in the amplified DNA and that they were the PCR limiting factors. Indeed, when the PCR was not driven to its final point, they were detected in the FAM and VIC channels (not shown).

3.2 Relative quantification of two mixed forms

To validate the strategy used for the analytical procedure, involving a PCR nested in a first one designed for avoiding amplification of genomic DNA, the B and AB cDNA forms, (chosen for the different migration times of their ssDNA, see Table 1), were mixed in different ratios from 2.5 to 97.5% (Figs. 3a and b). They were amplified by a first PCR, in order to obtain different mixtures of 250 bp-long fragments, then by a second nested PCR giving the ultimate fragment of 153 bp labeled with FAM and VIC fluorescent dyes on the forward and reverse strands, respectively. The ratios of B and AB forms, as calculated after CE from their RFU, were almost identical to the ratios of each cDNA form in the initial mixture (Figs. 3a and b). This was true for both ssDNA, the accuracy of the results given by one strand being identical to that of the other strand, with 0.02 <SD <1.5%. Therefore, under the amplification conditions employed here, the calculated relative proportion of one form to another reflected the exact initial proportions of the two given forms in a mixture. This was possible because both PCR were driven to their final points. Thus, identical results were obtained for more than 35 cycles (not shown). For determining to what extent these initial proportions could be detected, cDNA

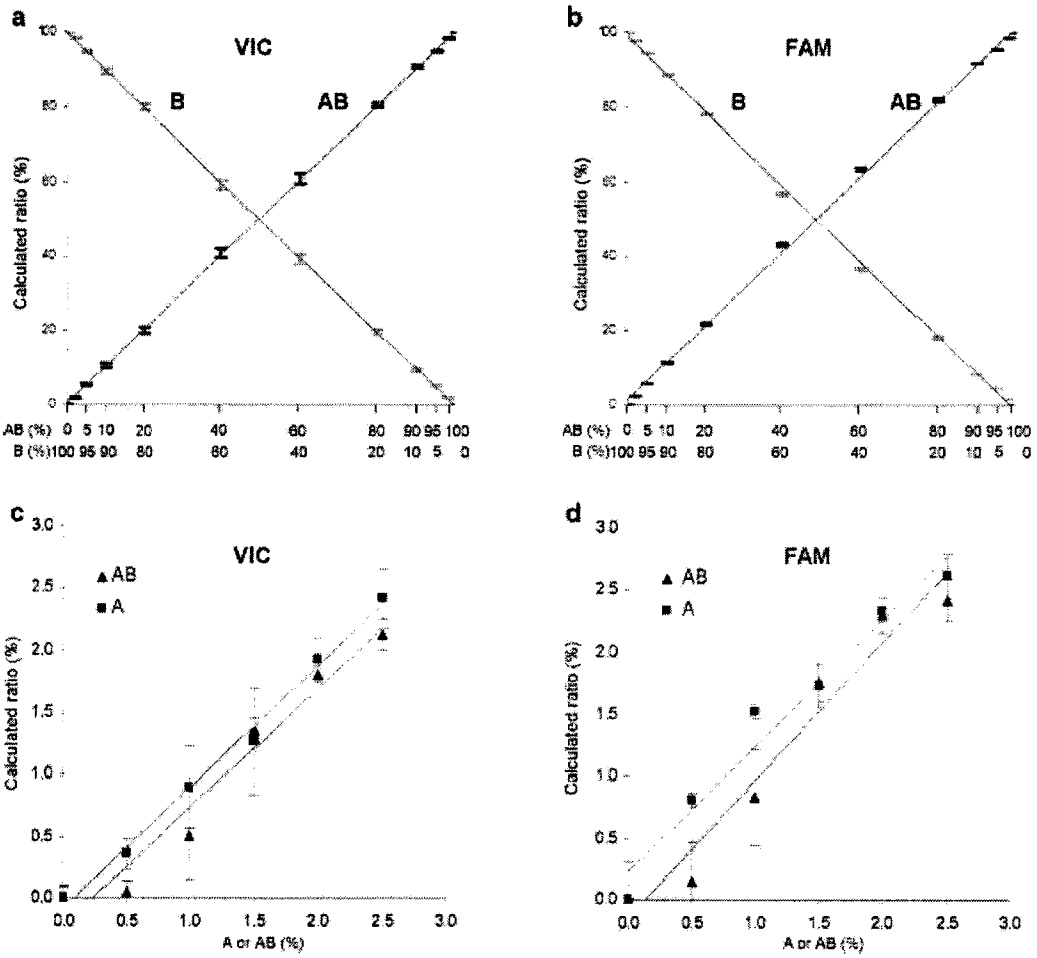

Figure 3. Relative quantification of B and AB forms mixed in different ratios. A total of 10 ng of plasmid DNA containing B and AB forms mixed in the indicated ratios (*x*-axis) was amplified by a first PCR, followed by a second PCR nested in the first one. VIC- and FAM-labeled strands were separated by CE and the amount of each separated strand was calculated from the area below each peak. (a) and (b) Calculated ratios of VIC- and FAM-labeled strands respectively, of B and AB forms as a function of the input mix (from 2.5/97.5% to 97.5/2.5%) as indicated; (c) and (d) calculated ratios of VIC- and FAM-labeled strands respectively, of B and AB forms as a function of the input mix (from 0.5/99.5% to 2.5/97.5%) as indicated. Values are the means ± SD of three independent experiments, and of two measures for each experiment, after separation by CE in duplicate.

forms A and AB were diluted into each other from 0.5/99.5 to 2.5/97.5% (Figs. 3c and d). In each case, as low as 1% was measured with a 0.06 <SD <0.39% for both VIC- and FAM-labeled ssDNA. These results demonstrate that, in the conditions used, the initial proportions of the different mixed cDNA forms are not modified by the two rounds of PCR. Therefore, assuming that the efficiency of the RT is identical for all 5-HT2cR mRNA forms present in the initial mixture of RNA, the initial proportions of these different mRNA forms should not be modified either by the two rounds of PCR.

3.3 Identification and relative quantification of the different forms in tissue samples

Total RNA was extracted from the FC and from the CP of four mouse brains. After RT, a 250 bp fragment of the 5-HT2cR cDNA was amplified between two primers that hybridize to sequences located in the two exons, bypassing the 93.5 kbp-long intron IV. In addition to extensive DNase treatment after RNA extraction, this strategy allows preventing any genomic DNA amplification. Identical amplification efficiencies of 5-HT2cR mRNA were always obtained after RT-PCR starting either from an oligo dT as primer for the RT, or from a specific primer hybridizing in exon VI (see Section 2.1) generally used in this study (not shown). A FAM- (forward strand) and VIC- (reverse strand) labeled 153 bp-long fragment was then amplified from the diluted (1/50) 250 bp PCR product. Here again, both PCR were driven to their final point, the FAM- and VIC-labeled primers being the limiting factors of the nested PCR. Figure 4a shows the typical electrophoresis profiles of the different ssDNA derived from the 5-HT2cR mRNA extracted from the FC and from the CP. The different peaks corresponding to the different VIC- and FAM-labeled fragments were matched to the 32 reference standards, the positions of which are given in Table 1. Percentages of the identified forms were calculated for each tissue (Fig. 4b). Twenty-three and 21 out of the 32 potential forms were identified in the FC and in the CP respectively, the least abundant representing about 0.5% of all 5-HT2cR

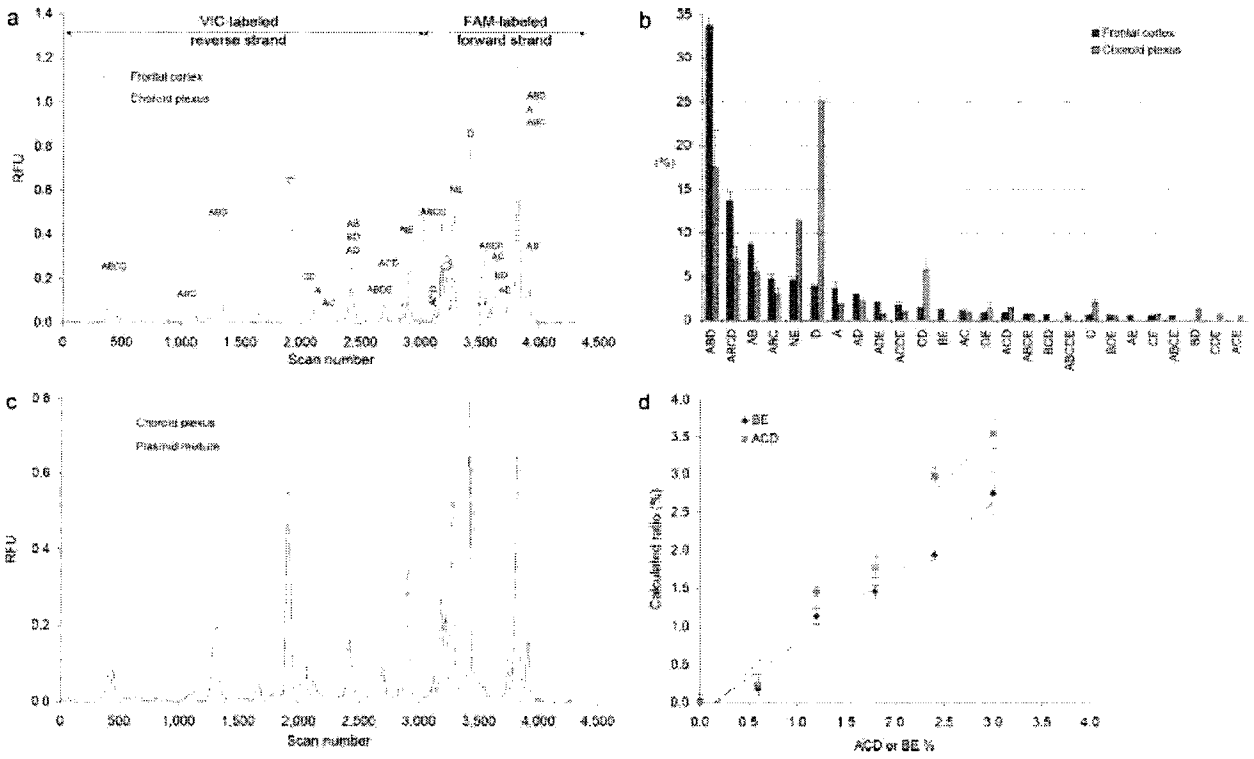

Figure 4. Analysis by CE of complex mixtures of VIC- and FAM-labeled cDNA separated on the basis of their SSCP. (a), (b) Identification and quantification of the different edited forms of 5-HT2cR mRNA in the FC and in the CP of mouse brain; VIC-and FAM-labeled amplified ssDNA were separated by CE in the conditions used to separate individual forms as described in the legend of Fig. 2; positions of each identified form are indicated above each peak for the VIC-labeled reverse strand and the FAM-labeled forward strand as specified for the two tissues in (a); the percentage of each identified form is given as indicated in (b) as the means ± SD of the values obtained from the analysis of four different mouse brains. (c) Superimposition of the electrophoresis profiles obtained by analysis of the cDNA derived from the CP and from a mixture of 22 plasmids in proportions identical to that determined by analysis of the CP. (d) Calculated ratios of ACD and BE forms as a function of the input forms added to the mixture of plasmids, as indicated. Values are the means ± SD of three independent experiments.

mRNA. The relative amounts of the different forms were significantly different in the two tissues (Fig. 4b). In particular, the ABD form was prevalent in the FC (34 *vs.* 17%), while it was the D form in the CP (25 *vs.* 4%). The native non-edited mRNA represented only 5% in the FC, but 12.6% in the CP, while the fully edited ABCDE form represented about 1% in the FC and was not detected in the CP. Assuming that a 5-HT2cR mRNA could not be edited on the E site alone (see Section 4), five additional forms were not found in any of these tissues (namely B, BC, ABE, BCE and BCDE). All these results were highly reproducible from one animal to another for a given tissue (Fig. 4b).

To test the accuracy of the method of quantification, 21 relevant cDNA were mixed in proportions identical to that determined by analysis of the CP. After the two rounds of PCR, the mixture of VIC- and FAM-labeled ssDNA was separated by CE, as was that of the ssDNA derived from the CP. Both electrophoresis profiles are extremely similar, as shown by their superimposition (Fig. 4c).

To determine to what extent a given form could be detected when diluted into a complex mixture, such as that derived from the FC or from the CP, either ACD or BE cDNA was added to a mixture of 21 cDNA. This mixture was the same as that used above for reconstructing the profile of 5-HT2cR mRNA editing in the CP, except that the ACD form was omitted, the BE form already being absent from the mixture. Whatever the relative amount of ACD or BE form added to this mixture, as low as 1% could be detected with a $0.07 < SD < 0.19$ for ACD and with a $0.33 < SD < 0.74$ for BE (Fig. 4d), even though both of them had either their VIC- or FAM-labeled strand comigrating with another ssDNA present in the mixture.

3.4 Accuracy of the method

For a given tissue, the profile of 5-HT2cR mRNA editing (Fig 4a) was obtained starting from 500 ng of total RNA. Because the overall procedure involves an RT-PCR followed by a second PCR nested in the first one, we verified to what extent the initial RNA could be diluted so that the relative proportions of the different 5-HT2cR mRNA remained constant. For this purpose, the entire procedure was carried out starting from 800, 400, 200, 100 and 50 ng total RNA extracted from the FC of one mouse. In each case, the relative amounts of the 14 different major forms representing more than 1% were compared. They were also compared with the percentages given by analysis of the FC of five different mice, each time starting from 500 ng of total RNA (Fig. 5a). The relationship between the results of the two groups of experiments gave an excellent coefficient of correlation ($r^2 = 0.994$). As long as the initial amount of total RNA was above 50 ng, the relative amounts of the different forms remained constant. Given that below this threshold, the different percentages obtained were no longer reproducible from one analysis to another (not shown), all quantifications were carried out starting with 500 ng of total RNA.

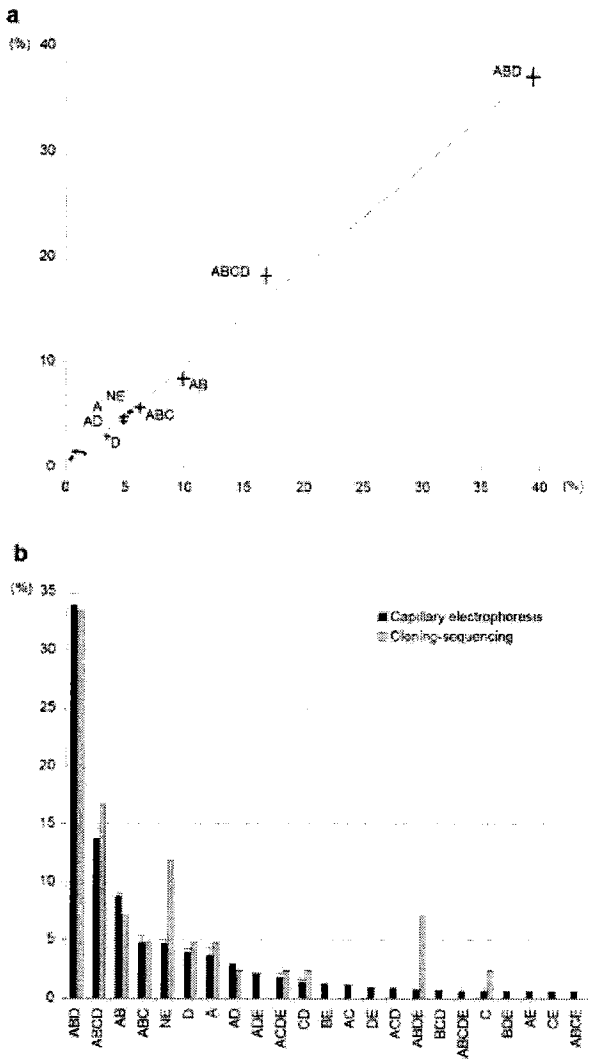

Figure 5. Accuracy of the method as shown in (a) by comparing the percentages of the 14 major forms obtained after dilution of the initial total RNA, from 800 to 50 ng (means ± SEM. with $n = 5$, on the *x*-axis), with the percentages given by analysis of the FC of five different mice, each time starting from a fixed amount of 500 ng total RNA (means ± SEM with $n = 5$, on the *y*-axis) giving $y = 0.954x + 0.0659$; and in (b) by comparing the percentages of mRNA edited forms calculated after sequencing 48 cDNA clones, starting from a mixture of RNA extracted from four mixed frontal cortices, to the percentages of cDNA measured after CE on the basis of their SSCP (means ± SD of the values given by analysis of four separate frontal cortices removed from four different mice).

Lastly, a 250 bp cDNA fragment was obtained starting from a mixture of total RNA extracted from the FC of four mice, and cloned as for the initial isolation of the different forms. Forty-eight clones were picked at random and sequenced. Twelve different forms were thus obtained, the percentages of which were determined. These results were compared to those obtained by the procedure described

above, which gave 11 additional forms ranging from 0.5 to 2.5% (Fig. 5b). More importantly, the percentage of each form was calculated as the mean of the results obtained for four separate FC removed from four different mice. The results clearly show that most of the less abundant forms were not detected by the cloning-sequencing procedure. In addition, some other forms appear with higher percentages than those given by the method described here (forms NE, ABDE and C in Fig. 5b).

4 Discussion

Finding a solution to the thorny problem of identification and quantification of the 32 putative forms of 5-HT2cR mRNA after A to I editing may allow solving any similar problem for any mRNA likely to be edited. Indeed, the five A subject to editing are in a row of 13 nucleotides and the percentages of the different forms of mRNA vary from almost 35% to less than 1%, as shown by analysis of the FC, making it very likely the most complex problem of this kind yet to be solved [1, 2].

The strategy used here for obtaining the mixture of cDNA involves an RT-PCR followed by a second PCR nested in the first one. The reverse primer used for the RT hybridizes either to a sequence located in exon VI or to the poly A tail, and the first PCR is achieved by use of sense primer hybridizing in exon IV and a reverse primer hybridizing in exon V, giving a 250 bp-long fragment without any risk of amplifying genomic DNA, thus avoiding overestimation of the NE form. In these conditions, assuming that the efficiency of the RT is identical for the putative 32 different mRNA forms generated by A to I editing, the first PCR should maintain the initial relative proportions of the different forms, since amplification was carried out to its final point in an identical way for all different forms present in a unique mixture. In addition, the product of the nested PCR also maintains these relative proportions, as clearly shown by the experiments depicted in Figs. 4 and 5, provided that the initial amount of total RNA used for the RT is above 50 ng. Therefore, using 500 ng of total RNA guarantees that the ultimate quantification takes account of the initial percentages of the different mRNA forms. In addition, results depicted in Fig. 4c show that the profiles of artificially mixed standards and actual experimental samples are basically identical; this indicates that the process of RT-PCR followed by secondary PCR does not alter the relative amounts of the different transcript variants for 5-HT2cR.

Separation of these different forms was achieved by capillary array electrophoresis on the basis of SSCP of a 153-bases fragment obtained after the second PCR. In the conditions described here, only the NE and E forms were not separated from each other. Setting up new conditions for separating them did not appear necessary to us because the E site has only a very low probability to be edited alone, the editing of other sites being a prerequisite to its own editing [1, 6, 12]. However, in case it would be necessary to separate the E from the NE form, this can be achieved by changing the parameters of the electrophoresis, but at the price of obtaining other unresolved forms (not shown). The relative quantification of each form was achieved by making the sum of the RFU given by both strands, using an algorithm developed for this purpose, thus overcoming the limitations of commercially available software [20]. This was realized by establishing 13 convenient migration standards and setting up electrophoresis conditions, allowing us to obtain a very high reproducibility in the retention time of each individual form, thus allowing for capillary-to-capillary and run-to-run variations [20, 21]. In addition, the method allows obtaining the mean value of each mRNA form of all 5-HT2cR mRNA present in a given sample. In contrast, sequencing at random a limited number of clones identifies only the forms of the same number of mRNA single molecules. Here, we have demonstrated in different ways that under these conditions, as little as 1% of a given form can be identified with certainty in a mixture of 23 or 21 forms of mRNA present in the FC or CP, after an electrophoresis of 105 min in 80 cm long capillaries, the fastest form migrating 12 min ahead of the slowest one. Therefore, the method described here opens the possibility to accurately determine variations in A to I editing upon drug administration as well as in human postmortem samples [11–14]. These variations are likely to be small, thus requiring extremely precise measurements for statistically significant results.

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5 References

[1] Valente, L., Nishikura, K., *Prog. Nucleic Acids Res. Mol. Biol.* 2005, *79*, 299–338.

[2] Schmauss, C., *Int. Rev. Neurobiol.* 2005, *63*, 83–100.

[3] Burns, C. M., Chu, H., Rueter, S., Hutchinson, L. K. *et al.*, *Nature* 1997, *387*, 303–308.

[4] Niswender, C. M., Sanders-Bush, E., Emeson, R. B., *Ann. N.Y. Acad. Sci.* 1998, *861*, 38–48.

[5] Niswender, C. M., Copeland, S. C., Herrick-Davis, K., Emeson, R. B., Sanders-Bush, E., *J. Biol. Chem.* 1999, *274*, 9472–9478.

[6] Fitzgerald, L. W., Iyer, G., Conklin, D. S., Krause, C. M. *et al.*, *Neuropsychopharmacology* 1999, *21*, 82S–90S.

[7] Wang, Q., O'Brien, P. J., Chen, C.-X., Cho, D.-S. C. *et al.*, *J. Neurochem.* 2000, *74*, 1290–1300.

[8] Marion, S., Weiner, D. M., Caron, M. G., *J. Biol. Chem.* 2004, *279*, 2945–2954.

[9] Herrick-Davis, K., Grinde, E., Niswender, C. M., *J. Neurochem.* 1999, *73*, 1711–1717.

[10] Price, R. D., Weiner, D. M., Chang, M. S., Sanders-Bush, E., *J. Biol. Chem.* 2001, *276*, 44663–44668.

[11] Niswender, C. M., Herrick-Davis, K., Dilley, G. E, Meltzer, H. Y. *et al.*, *Neuropsychopharmacology* 2001, *24*, 478–491.

[12] Sodhi, M. S., Burnet, P. W., Makoff, A. J., Kerwin, R. W., Harrison, P. J., *Mol. Psychiatry* 2001, *6*, 373–379.

[13] Gurevich, I., Tamir, H., Arango, V., Dwork, A. J. *et al.*, *Neuron* 2002, *34*, 349–356.

[14] Sodhi, M. S. K., Airey, D. C., Lambert, W., Burnet, L. P. W. *et al.*, *Mol. Pharmacol.* 2005, *68*, 711–719.

[15] Kishore, S., Stamm, S., *Science* 2006, *311*, 230–232.

[16] Iwamoto, K., Bundo, M., Kato, T., *RNA* 2005, *11*, 1596–1603.

[17] Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T., *Proc. Natl. Acad. Sci. USA* 1989, *86*, 2766–2770.

[18] Maekawa, M., Sugano, K., Ushiama, M., Masuda, T. *et al.*, *Biochem. Biophys. Res. Commun.* 1996, *223*, 520–525.

[19] Liu, Z. J., Peng, W.-C., Yang, X., Huang, J.-F. *et al.*, *J. Chromatogr. B* 2003, *793*, 405–412.

[20] Andersen, P. S., Jespersgaard, C., Vuust, J., Christiansen, M., Larsen, L. A., *Hum. Mutat.* 2003, *21*, 455–465.

[21] Kourkine, I. V., Hestekin, C. N., Barron, A. E., *Electrophoresis* 2002, *23*, 1375–1385.

